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OUTLINES OF BIOCHEMISTRY

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The Organic Chemistry and the Physico-Chemical Reactions of Biologically Important Compounds and Systems

BY

ROSS AIKEN GORTNER

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THIS VOLUME IS DEDICATED TO

MY COLLEAGUES

TO THOSE PERSONS, PAST AND PRESENT, WHO HAVE BEEN ASSOCIATED WITH ME ON THE STAFF OF THE DIVISION OF AGRICULTURAL BIOCHEMISTRY IN THE UNIVERSITY OF MINNESOTA; FOR TO EACH AND EVERY ONE I OWE MUCH, AND IT IS ONLY THE SPLENDID COOPERATIVE SPIRIT OF THIS GROUP THAT HAS MADE THIS VOLUME POSSIBLE.

PREFACE

"When I have the honor of being consulted by a young man who has not yet found himself intellectually but who is filled with the desire to devote his life to some branch of medicine, be it clinical medicine, pathology, hygiene, bacteriology, physiology or pharmacology, my advice always is, 'Study chemistry at least three years. Try with all your power to master enough of this great science to start you on your career.'"

John J. Abel.

It is rather generally agreed among the scientists that the actions and reactions of a biological organism are expressions of the energy relationships due to chemical and physico-chemical processes taking place within the cells and tissues which comprise the organism.

The biological organism can be looked upon as a complex system of chemical constituents, composed mainly of proteins, carbohydrates, fats and lipides, mineral elements, and water, which are organized by the mysterious forces which we call "life," and the actions and reactions of this protoplasmic mass are in turn determined by the energy interchanges of molecular transformations and surface and interfacial forces.

In most of the universities of America the development of the field of biochemistry has been left very largely to the group interested in the medical aspects. Accordingly, in a very large measure the biochemistry of the American universities is not biochemistry in its strictest sense, but rather leans more and more toward the field of human pathology. Most medical school biochemistry could be more correctly designated as human pathological chemistry.

It is obvious that there should be strong departments of physiological chemistry associated with the medical schools. However, it should likewise be recognized that there is a necessity for a study of the fundamental reactions underlying the broader field of biology, the primary object of which is to study and investigate the chemical and physico-chemical reactions which take place in the normal biological organism, whether that organism be animal or plant.

The greatest advance in the biological sciences can take place only when the chemists are fully aware of certain of the biological problems and the biological point of view, and only when the biologists appreci-

ate the assistance which chemical knowledge and chemical technic can offer to the solution of the major problems.

When, in 1913, Professor R. W. Thatcher was called to the University of Minnesota, he inaugurated a course of lectures on "Phytochemistry" and taught the course until he became Dean of the Department of Agriculture in 1917, at which time Dr. C. A. Morrow assumed charge of the lecture work in "Phytochemistry" and supervision of the parallel laboratory course in "Biochemical Laboratory Methods."

Dr. Morrow remained in charge of both courses until his unfortunate illness in the spring of 1922, following which illness the responsibility for the lecture work was assumed by the present author, and upon resumption of active duties Dr. Morrow was left free to devote his entire energies to the development of the associated laboratory phases of the work. His success in this task is attested by the practical completion of the manuscript of the laboratory manual, "Biochemical Laboratory Methods," which appeared posthumously from the press of John Wiley & Sons, Inc., in 1927.

The course of lectures upon which the present volume is based must not therefore be regarded as the product of a single individual, for it embodies primarily the efforts of three men who have actually taught the course, and in addition the advice, cooperation, and criticism of all other persons who from time to time have been members of the Staff of the Division of Agricultural Biochemistry.

During the year 1925–26 the present author prepared a series of mimeographed lecture outlines as an aid to the students in the classroom. These outlines were somewhat revised in the summer of 1927 and issued to the students in bound mimeographed form. The present volume follows, in general, the topics as presented in these outlines, although the scope of the field has been greatly expanded.

All of the reactions and interactions which we call life take place in a colloid system, and the author believes that much of the "vital energy" can in the last analysis be traced back to energies characteristic of surface films and interfaces. This belief is the justification for the detailed consideration of colloid systems which forms the first section of the volume.

In the subsequent sections dealing with proteins, carbohydrates, fats, etc., those organic compounds characteristic of living tissues, particular attention has been paid to structural organic chemistry and organic and physico-chemical reactions. No attempt has been made to produce a "handbook" of biochemical compounds or a "descriptive biochemistry" detailing the chemical properties, crystal structure, etc., of the various compounds. There are already many volumes covering

these fields, and no good purpose would be served by adding to the list.

The purpose of the present volume is that those students who are interested in biological phenomena may have an insight into the roles which organic chemistry and physical chemistry play in living processes. It may be regarded as an attempt to interpret some of the reactions characteristic of the normal cell. Although a great many of the illustrations have been drawn from plant material, it must be remembered that in the last analysis the chemistry of the cell is essentially the same both for plants and for animals. There is no sharp distinction between "phytochemistry" and "zoochemistry." The same general reactions of protoplasm apply to both. If the student interested in some pure or applied field of biology or of chemistry is assisted, ever so little, in the clarification of the problems in his special field, then the object of the author will have been attained.

Toward this end the exact titles have been included in the literature citations. With but few exceptions (and those are noted in the text) the citations have been checked back against the original publication so as to preclude bibliographic errors. A sincere attempt has been made to give to other investigators the proper credit for data or for ideas which have been utilized. On the other hand, the text does not pretend to be an exhaustive treatment of the subjects, nor is the literature cited necessarily in an historical sequence. It is recognized that in many instances the references cited are not the first publications on the subject, but it is felt that the references which are cited contain something which should be called to the attention of the reader. The author may perhaps be pardoned for the numerous references to work done in his own laboratories, for obviously he is most familiar with the details of such work and the conditions under which it was carried out.

In conclusion, the author wishes to thank his colleagues, Dr. J. J. Willaman and Dr. Leroy S. Palmer, who have contributed Chapters XXVII and XXXIV, respectively; The Williams & Wilkins Company, and Prof. W. Mansfield Clark; the editors of the Journal of Biological Chemistry; The Chemical Catalog Company, Prof. Harry N. Holmes, Prof. O. L. Sponsler, and Prof. W. H. Dore; The Carnegie Institution of Washington, and Dr. D. T. MacDougal; Carl Zeiss, Inc.; Dr. Karl Mez; Prof. John J. Abel; Prof. E. F. Burton; Jerome Alexander; Prof. Francis Lloyd; Dr. Robert Newton; Dr. G. E. Holm; Dr. P. V. Wells; Dr. William Robinson, and others for permission to use copyrighted material or for photographs or data.

Parts of the manuscript have been carefully read and criticised by my colleagues, Dr. W. M. Sandstrom, Dr. J. J. Willaman, Dr. L. S.

Palmer, Dr. C. H. Bailey, Dr. W. B. Sinclair, Dr. David R. Briggs, and Mr. Chas. F. Rogers. To them I express my thanks.

And lastly I wish to express my deepest appreciation to Miss Rachel Rude for invaluable assistance in the task of preparing the final manuscript and in proofreading.

Ross Aiken Gortner.

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ABBREVIATIONS AND SYMBOLS

The abbreviations used in the literature citations are, in general, those used in *Chemical Abstracts*. The symbols used in the mathematical formulæ follow, insofar as possible, those recommended in *International Critical Tables*, Vol. I, pp. 16–17.

I

COLLOIDS

"The colloidal is, in fact, the dynamical state of matter; crystalloidal being the statical condition. The colloid possesses ENERGIA. *It may be looked upon as the probable primary source of the force appearing in the phenomena of vitality."*

THOMAS GRAHAM (1861)

"The colloid field today presents the most promising realm in medical research."

W. J. MAYO (1928)

OUTLINES OF BIOCHEMISTRY

CHAPTER I

THE COLLOID STATE OF MATTER

It seems appropriate to begin the study of biochemistry with a consideration of the colloid state of matter, because in the last analysis many of the reactions of biological systems are dependent upon the colloidal phenomena operating in such systems. It is recommended that the beginner read "Theoretical and Applied Colloid Chemistry" by Wo. Ostwald, as translated by Martin Fischer.[1] This volume represents five lectures delivered by Professor Ostwald during a tour of the United States and Canada in the winter of 1913 and 1914. These lectures were prepared for the average reader, rather than for the specialist, and accordingly furnish a bird's-eye view of certain of the general phenomena characteristic of the colloid state. Incidentally the reader will become acquainted with some of the special nomenclature in this field.

We often hear the statement that such and such a material is a colloid. Such a statement is not strictly correct. Colloid phenomena deal with a *state* of matter, not a kind of matter. Early workers in this field published many papers indicating that certain materials could under certain specific conditions of manipulation be brought into the colloidal state. We now know that, using the proper technic, any material can be brought into the colloidal state. Consequently there is no sharp line of demarcation between substances which can be regarded as colloids and substances which are generally non-colloids.

The first real work in the field of colloid chemistry was due to the activities of Thomas Graham (1805–1869). Graham's early important contributions were published in the period of 1861–1864, and it is to him that we owe the first use of the term, colloid, derived from the Greek, κολλα, Kolla, meaning glue, and ειδος, Eidos, meaning like, the term colloid thus meaning glue-like, or the entire class being named from a single component.

[1] John Wiley & Sons, Inc., New York, 1917.

Graham believed that there was a sharp distinction between those materials which behaved as colloids and those which were characteristically crystalloids. In the former class he placed glue, gelatin, dextrin, the various gums, proteins, etc., and in the latter class such substances as sucrose, glucose, sodium chloride, etc.

As noted above, we now know that there is no sharp distinction between colloids and crystalloids, and as we shall see later, any substance, even including such substances as sodium chloride, can by proper technic and by the proper choice of medium be brought into the colloidal state. For example, some substances may be colloidal in one liquid medium and truly crystalloidal in another. Thus, tannin dispersed in water gives rise to a colloidal system, whereas it dissolves in acetic acid to form a crystalloidal solution. Many other substances show similar behavior. *Only the physical properties of the resulting system will show whether or not a given material is colloidal or crystalloidal.*

As has already been indicated, a substance cannot be strictly spoken of as a colloid, for the colloid implies (1) a state of matter, and (2) at least two components or phases. Thus, we have not a colloid material but rather colloidal systems.

Water has three states, vapor, liquid, and solid. Each of these may exist in colloidal systems. Thus, for example, Barnes[2] has discussed certain natural phenomena produced by colloidal water and colloidal ice, and we shall have occasion later to consider certain instances where water vapor is involved.

Inasmuch as a colloid system is a heterogeneous system, it is necessary to distinguish between the substance which is dispersed and the medium in which the material is dispersed. Various terms have been employed by the different writers. Thus, for example, some authors speak of the *disperse phase* and *dispersions medium;* others of the *discontinuous phase* and the *continuous phase;* others of the *internal phase* and the *external phase;* and still others of the *micelles* and the *intermicellar liquid.* The last group of terms appears to be coming more and more into general use and in some respects appears preferable to the other groupings.

Martin Fischer has given us a definition of a colloidal system which is fairly satisfactory. He states that "colloid systems result whenever one material is divided into a second with a degree of subdivision coarser than molecular." This definition, while approximate, is not strictly true, although for the beginner it perhaps illustrates in a desirable manner a common distinction between crystalloidal and colloidal systems.

[2] Barnes, Howard T., Colloidal Water and Ice, Colloid Symposium Monograph, Vol. III, pp. 103–111, Chemical Catalog Company, New York (1925).

The definition could probably be restated with minor changes so as to hold rigidly. Such a restatement would yield the following definition: *Colloidal systems result whenever one material is divided into a second with a degree of subdivision either (a) coarser than molecular or (b) where the micelles exceed* 1–1.5 $m\mu$ *in diameter.*[3] A graphic representation of the field of colloid chemistry is shown in the following diagram:

Molecules and ions	Colloids	Matter in Mass
Not Visible in Ultramicroscope	Visible in Ultramicroscope	Visible in Microscope
	$1m\mu$ 0.1μ	

It will be noted that an arbitrary boundary has been placed at $1m\mu$ $\left(\frac{1}{1{,}000{,}000}\text{ of a millimeter}\right)$ defining the lower limit of the colloid realm, and another arbitrary boundary at approximately $0.1\mu\left(\frac{1}{10{,}000}\text{ of a millimeter}\right)$ as defining the upper limit of the colloid realm. It cannot be too strongly emphasized that *these are arbitrary boundaries.* However, they seem to have been rather wisely chosen. The recent work of physicists and physical chemists has indicated that, in general, molecules and ions which are strictly crystalline in solution rarely exceed $1m\mu$ in diameter. In some instances, for example, in the case of egg albumin, we have truly monomolecular solutions which at the same time exhibit certain properties of colloidal systems. This phenomenon is not in disagreement with the second part of our definition of a colloid system noted above. We are probably dealing in such cases with massive molecules, perhaps 5 to $10m\mu$ in diameter.

The upper limit of the colloid realm, 0.1μ in diameter, was chosen because this represents the extreme range of the ordinary microscope, using an oil immersion objective. The colloid realm is thus concerned with systems containing subdivisions of matter lying between the limits of visibility of the usual laboratory microscope and those solutions which are strictly crystalloidal. It must be understood at the outset that the properties of the colloidal systems do not strictly coincide with these arbitrary boundaries but that *there is a continual gradation in properties from truly crystalloidal systems to coarsely divided suspensions, and that the optimum zone of colloidality lies somewhere near the center of the colloid realm, as noted in the diagram above.* In other words a coarsely

[3] For the justification of using $m\mu$ instead of $\mu\mu$ to designate millimicron see "A Protest" by H. S. Uhler, *Science* 65 : 232–233 (1927). Much of the older literature uses the symbols $\mu\mu$ to express millimicron.

divided suspension will show to a minor degree certain of the phenomena characteristic of the colloidal state, and in the same way certain of the phenomena of the colloidal state reflect in a minor degree the characteristics of true solutions.

Siedentopf and Zsigmondy classify the three states of matter noted in the above diagram as *microns* visible in the microscope, *submicrons* or *ultramicrons* visible in the ultramicroscope, and *amicrons* not visible in the ultramicroscope. According to their terminology all systems showing characteristic colloidal properties contain submicrons. We are probably nearer the truth when we place the lower limit of colloid systems at $1m\mu$, than when we place the upper limit at 0.1μ, and there has been a general tendency of recent years to raise the upper limit to perhaps 0.5μ, inasmuch as fine suspensions possess to a large degree certain characteristic properties of colloidal systems.

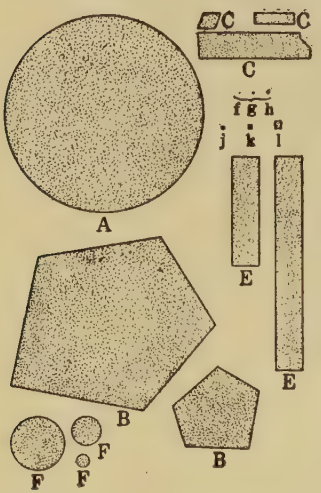


FIG. 1.—*A* = red blood cells; *B* = starch grains; *C* = clay particles; *E* = *Anthrax bacilli*; *F* = *Cocci*; *fgh* = colloidal gold (f = 10mμ); *jkl* = gold coagula. (After Zsigmondy.) By permission from Alexander's (1924) "Colloid Chemistry."

Perhaps everyone has viewed a red blood corpuscle under the microscope and accordingly has some idea of the relative size of a red blood corpuscle as compared with other familiar objects. Figure 1 illustrates the size relationships of some rather common materials as compared with the size of colloidal particles. It should be noted, however, that the smallest colloidal particle noted at (f) is ten times the diameter of the particles which fix the lower limit of the colloidal realm.

According to Wo. Ostwald one can conveniently classify colloid systems into eight great groups:

1. Solid-in-solid.—Examples of such systems are the ruby glass of the cathedral windows which is a colloid system of metallic gold dispersed in glass; blue rock salt, a colloid system of sodium dispersed in sodium chloride; the black diamond which is carbon dispersed in crystalline carbon. The colors of many precious stones are probably examples of solid-in-solid colloidal systems. This type of system is important in metallurgy, but from the biological standpoint is relatively unimportant.

2. Solid-in-liquid.—Faraday, in 1857, exhibited before the Royal Society a brilliant red liquid which he had prepared by reducing an aqueous

solution of auric chloride. Inasmuch as this liquid showed a beautiful red color by transmitted light and a golden sheen by reflected light, he suggested that the color might be due to particles of solid gold suspended in the liquid. This experiment was forgotten for many years, but we now know that Faraday had prepared one of the first examples of a solid-in-liquid colloidal system. His original preparations are still in the museum of the Royal Society.

Wo. Ostwald has given to this class of colloid systems the specific name of *suspensoids*, and this term is used rather generally in the literature. The definition of "a solid-in-liquid" does not at all times tell the true story. In the case of gold-in-water systems we have a true solid-in-liquid, but it is easy to prepare a system having similar properties by the dispersion of an oil in water. Both preparations would according to Ostwald's terminology be called suspensoid systems. Perhaps a better terminology would be a *lyophobic* system, and a better definition for solid-in-liquid would be one suggested by Martin Fischer, namely, *a system in which the disperse phase is insoluble in the dispersions medium and the dispersions medium does not dissolve in the disperse phase.* Thus, for example, we may have systems of gold and water, sulfur and water, rubber and water, sodium chloride and alcohol, etc. This type of system is of rather general importance in biochemical studies, and certain of its characteristics will be considered in greater detail in the following pages.

3. Solid-in-gas.—In this system we are dealing with such phenomena as the smoke-vapors of ammonia and hydrochloric acid, the "blue haze" of the forest fire areas that hangs for weeks in the air without settling, and the "blue" smoke of the cigarette. The darker colored smokes usually contain the more coarsely divided suspensions of carbon. When these larger particles have settled out, the blue colloidal haze still persists and forms a very stable system. Such systems are of negligible importance from the biological standpoint but from the military standpoint constitute a rather important field of study involving smoke screens, etc., for the reason that one desires the maximum density of smoke which can be formed from the minimum amount of original material, the degree of subdivision thus playing a very important role.

4. Liquid-in-solid.—The principal examples of this system are to be found in minerals and gems. Characteristic examples are the opal and the pearl. The opal is a system of silicon dioxide and water, the pearl a system of calcium carbonate and water. An opal rather readily loses its "fire" and the pearl its "life" or lustre if kept for a considerable period of time in a dry atmosphere. Instances have been known where valuable pearls placed for years in a safe-deposit box have

been rendered practically valueless. Both the opal and the pearl are most beautiful when worn often near the skin, *i.e.*, in a region of fairly high humidity tending to keep the equilibrium amount of water in the gem.

5. Liquid-in-liquid.—This is the class that Wo. Ostwald calls the *emulsoids* and which is generally referred to as the *lyophilic colloids.* Here again the "liquid-in-liquid" terminology is not strictly accurate. A far better definition is that of Martin Fischer who defines this group as "*a system in which the disperse phase and the dispersions medium are mutually more or less soluble one in the other.*" This indicates that *hydration* (when water is the dispersions medium) or *solvation* (a term which applies to any dispersions medium) takes place. Typical examples are gelatin and water. Gelatin swells in water; some of the gelatin disperses in the water, and a considerable amount of the water "dissolves" in the gelatin. The gelatin may be a liquid particle or it may be a solid particle. We do not know whether either of these conditions actually exists, but we do know that gelatin in water is *hydrated,* and that is the important point. Gelatin in water is a lyophilic (solvent-loving) system. Gelatin in alcohol or benzene is a lyophobic system. Rubber in alcohol or benzene is a lyophilic system, in water a lyophobic system. This class of lyophilic colloids is by far the most important class that we shall meet, in regard to biological problems, and will be referred to in general as the lyophilic colloids.

6. Liquid-in-gas.—Fogs, mists.—This class is important from the standpoint of meteorology. In the case of fogs and mists we probably deal with a solid-in-gas, with water particles condensed on the solid surfaces. Owens[4] has made the following calculations in regard to the conditions which bring about fog in the London area. He finds that on a clear day there is approximately 1 milligram of solid material per cubic meter of air. In a dense fog the amount of solid material rises to the neighborhood of 5 milligrams of solid material per cubic meter of air. This does not seem a very large amount, but over the London area it amounts to 193 tons of solid material on the 120 square miles of area to a height of 122 meters. The size of the solid particles varies from 0.00013 to 0.00026 millimeter in diameter. The water film condensed on the surface of these solid particles may be as great as 0.0014 millimeter in thickness. Owens notes that at 6 a.m. the air above London may be perfectly clear and at 9 a.m. there may be a dense fog, and he ascribes the onset of the fog to the smoke rising from the fires of the

[4] Owens, Seventh Report of the Committee for the Investigation of Atmospheric Pollution, No. 249, Meteorological Office, Air Ministry, London, 1922. (See *Science* 55 : 596–597, 1922.)

homes and factories. In the Ninth Report on Atmospheric Pollution[5] for the year ending 1923, there is a further amplification of the conditions under which fogs develop. It is pointed out that on a clear day there are approximately 100 particles of solid per cubic centimeter of air, whereas in a dense fog there may be as many as 80,000 particles of solid per cubic centimeter. Incidentally it may be noted that 10,000 particles of solid per cubic centimeter are equivalent to a weight of 1 milligram per cubic meter. The diameters of the particles range from a maximum of 1.5μ down to the ultramicroscopic particles. In one dense London fog a count of 53,000 particles per cubic centimeter of air was made. It may be noted that the solid content of air is a rather important biological problem. Thus under London conditions a person would inhale 500 billion particles in a period of twenty-four hours, or enough particles to make a string 250 miles long. A considerable part of these is removed by the cilia and cells of the lungs, but a very substantial part is retained. Pathologists are often able from post-mortem examinations to differentiate between city dwellers and people who have dwelt under rural conditions. Adults from rural districts usually have pink lungs, whereas city dwellers almost invariably have lungs which are dark brown and in many instances as black as charcoal. In the pathological museum of the University of Chicago there are exhibits of city dwellers' lungs and of material removed from such lungs. In one instance there is a test-tube almost completely full of black carbon which was removed from one lung of a city dweller. The corresponding opposite lung is on exhibit and is dense black.

London is cited in the above examples as a typical example of a modern city. The amount of solid material deposited from the air per month in certain of our own cities has been recorded[6] as follows:

		Tons
Pittsburgh,	April, 1923	115.6
	April, 1912	98.7
	April, 1916	109.8
St. Louis,	April, 1916	59.6
Cincinnati,	April, 1916	52.0

7. Gas-in-solids.—From the biological standpoint this field is relatively unimportant. From the standpoint of mineralogy, metallurgy, and

[5] Ninth Report of the Committee for the Investigation of Atmospheric Pollution. Report on Observations in the year ended March 31, 1923, Meteorological Office, London (1924). Abstracted in *Science*, 60 : 136 (1924).

[6] Meller, H. B., Economy through Smoke Abatement, *Ind. Eng. Chem.*, 16 : 1049–1051 (1924).

industrial chemistry it offers a fruitful field of research. The form in which gas is present in solids is still in many instances a debatable question. In some minerals gas probably occurs in the form of very finely divided bubbles, the bubbles perhaps being of colloidal dimensions. In other instances the gas is probably adsorbed or "fixed" on the surface of the solid material. This is undoubtedly the form in which helium occurs in the mineral, Pitchblende. Many solid materials possess the property of condensing or adsorbing gas upon their surfaces, and a great variety of industrial applications have been based upon this phenomenon. Possibly such systems could be logically spoken of as gas-in-solid systems. However, this is not the place for an extended discussion of such systems, which will be reserved for consideration later under adsorption.

8. Gas-in-liquid.—Such systems are generally spoken of as foams or systems in which gas bubbles are finely divided and suspended in liquids. The liquid in which the gas bubbles are suspended is usually not a pure material but is more often already a colloidal system containing lyophilic colloids. Typical foams are produced when egg white is beaten or when cream is whipped, the lyophilic colloids which are present serving to stabilize the resulting foam. Biologically this group is of only slight importance, but industrially it may be of extreme importance. In many instances foams which are extremely difficult to break develop in industrial processes. Accordingly a study of such systems is necessary in order to devise methods for the destruction of foams at points where they are undesirable.

9. Gas-in-gas.—The foregoing eight classes of colloidal systems have all been realized experimentally and all occur in nature. The ninth hypothetical system, gas-in-gas, is theoretically impossible to attain, inasmuch as gas is always regarded as occurring in the molecular state, so that large aggregates of molecules are theoretically impossible, for no known gas molecule is of colloidal dimensions. Possibly if someone were to work with mixtures of gases at their critical state, this system might be experimentally realized. It is doubtful, however, whether it would be of any technical importance. Certainly it would have no biological significance.

Emulsions.—An additional system which should be considered in a study of colloid chemistry is the emulsions. Emulsions are not necessarily truly colloidal systems, if we limit our definition to the size of the particle concerned. Emulsions may be defined as systems of oil dispersed in water or as systems of water dispersed in oil, although in many instances the dispersions medium contains a lyophilic colloid to assist in stabilizing the emulsion. Very dilute emulsions could be classified as liquid-in-liquid systems, providing all of the particles fell

within the range of diameters characteristic of colloidal systems. Most emulsions, however, contain oil or water droplets very much larger than even the 0.5μ, which forms the upper limit of the truly colloidal field. Mayonnaise, milk, and egg yolk are typical examples of emulsions with which everyone is familiar. In each one of the examples we are dealing not only with oil droplets suspended in an aqueous medium, but we have present at the same time lyophilic colloids in the form of proteins which stabilize the emulsion, so that a study of emulsions involves not only a study of the dispersions medium and the disperse phase, but likewise, and probably of greater importance, an investigation of the nature and properties of the lyophilic colloid which acts as the stabilizer.

Protoplasm has been spoken of as an emulsion. Certainly there are droplets of fats and oils in living protoplasm which are stabilized by the lyophilic colloids which are present. It is equally true that there are solid particles in living protoplasm, so that protoplasm can be looked upon as a complex colloid system, the dispersions medium being water and the disperse phases consisting of lyophilic colloids, lyophobic colloids, and microscopically visible fat droplets in the form of an emulsion as well as other microscopically visible particles.

Nomenclature.—It is necessary at this point to consider a few general terms of special nomenclature.

We speak of liquid colloidal systems as *sols*. A sol may be defined as a colloidal system which to the eye appears as a solution, *i.e.*, it is fluid and appears to be homogeneous. It can be more or less readily poured from one beaker to another, but it differs from a solution in that the sizes of particles suspended in the liquid are of colloidal dimensions. Occasionally in speaking of sols, it is desirable to define the dispersions medium. Thus we have such terms as hydrosols where water is the dispersions medium, alcosols, benzosols, etc., with organosols as a special term limited to organic solvents as a dispersions medium.

We speak of more or less rigid colloidal systems as *gels*. A gel may be defined as a colloidal system possessing more or less the properties of a solid. Gels logically fall into two general subdivisions.

1. The true gels or "jellies," as some prefer to call them, are formed by the characteristically lyophilic colloids and may be represented by such familiar materials as gelatin, thick custards, the ordinary fruit jellies and jams of the household, muscle tissue, heat-coagulated egg white, etc., and from the biological standpoint constitute the important group of gels.

2. Another subdivision, sometimes referred to as gels, is the precipitates which are formed by the coagulation of lyophobic colloid systems. These are preferably referred to as *coagula* rather than as gels, although

there is no general agreement in regard to this distinction in nomenclature. When a gold hydrosol is precipitated by the addition of an electrolyte (*vide infra*), a purplish precipitate is thrown down. This precipitate is relatively bulky, and when this precipitate is ignited, it is often found that an almost unweighable amount of gold is actually present. The precipitate contains the gold particles originally dispersed in the form of a sol, and adhering to these coagulated particles is a relatively large amount of the dispersions medium (water). Inasmuch as such a precipitate does not show, in general, properties similar to those exhibited by the "jellies," it seems preferable to differentiate between the two types and to refer to the precipitates formed in lyophobic systems as *coagula*.

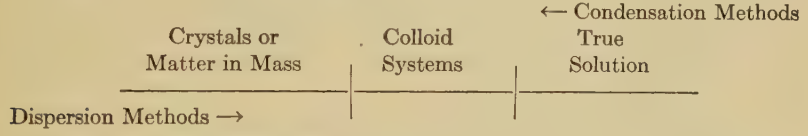
In the gels as in the sols, we use such special terminology as hydrogels, alcogels, benzogels, organogels, etc.

The terms lyophilic and lyophobic have already been defined and are general terms referring to the affinity or lack of affinity of the disperse phase for the dispersions medium. A lyophilic system in general produces a gel which contains a high percentage of the dispersions medium. A lyophobic system on the other hand yields a coagulum which contains a relatively small amount of the dispersions medium. There are a few exceptions to the above rules (for example, di-benzoyl-*l*-cystine gel) which will be taken up later.

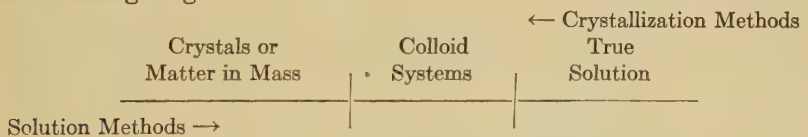
CHAPTER II

METHODS OF PREPARATION

THE methods of preparation of colloidal systems are referred to in the literature under two separate systems of nomenclature. We have the classification due to Svedberg, which is used by Wo. Ostwald, as illustrated in the following diagram:



Or we have the classification due to Von Weimarn, as illustrated in the following diagram:



It will be noted that these classifications are essentially identical, the significant feature of both being to illustrate the fact that there is a continuous gradation from matter in mass to true solutions. Every crystal which grows in a true solution has at one time possessed dimensions which would bring it within the colloid realm. Likewise every crystal which dissolves to form a true solution must eventually yield a particle of colloidal size before it completely disappears into true solution. Either Svedberg's or Von Weimarn's classification is satisfactory.

To prepare a stable colloidal sol one must stop when the particles are within the colloidal realm. It is also reasonably desirable to have fairly high concentration of the disperse phase in the dispersions medium.

When the condensation methods are employed, the stability and the concentration of the resulting system depend upon two factors, (a) the velocity of the formation of nuclei, and (b) the rate of crystal growth. Von Weimarn has expressed these two phenomena in some rather general formulae.

$$W = K \frac{\text{Condensation pressure}}{\text{Condensation resistance}} \qquad (1)$$

where (W) is the velocity of nuclei formation and (K) is a constant.

If (C) is the total amount in solution and (L) is the solubility, $(C - L)$ is the condensation pressure or the supersaturation, and (L), the solubility, is the condensation resistance. The formula then becomes

$$W = K\frac{(C - L)}{L} \tag{2}$$

This formula means that there is a certain tendency for the material to remain in a supersaturated solution expressed by condensation resistance and that the velocity is dependent upon the ratio of these two factors to each other and to a constant. It is obvious that the more nuclei that are formed, the greater will be the surface area of the disperse phase, and likewise the smaller will be the diameter of the resulting particles per unit weight of material. To form a stable colloidal system it is, therefore, desirable to have as great a number of nuclei formed as is practicable.

Once the nuclei are formed, they grow according to the Noyes-Nernst formula for crystal growth.

$$V = \frac{\Delta}{l} S(C - L) \tag{3}$$

where V = velocity of growth;
Δ = diffusion coefficient;
l = length of diffusion path;
S = surface area of disperse phase;
C = concentration of solution;
L = solubility of disperse phase of a given size;
$(C - L)$ = absolute supersaturation.

As illustration let us take the examples of silver chloride and sodium chloride; for sodium chloride $(C - L)$, the supersaturation, may be relatively great, and $\left(\frac{C - L}{L}\right)$ or the excess of sodium chloride in a solution of the very soluble sodium chloride will make for a low velocity of nuclei formation and for the formation of relatively large crystals of sodium chloride. Accordingly a hydrosol of sodium chloride is not attainable. With the same values for $(C - L)$ for silver chloride we get at once a dense, curdy precipitate (a gel or coagulum) of silver chloride. In the case of sodium chloride (L) was large, and slow crystallization resulted. In the case of silver chloride (L) is extremely small

and instant precipitation occurs. If instead of using an aqueous medium we had formed the sodium chloride by the interaction of sodium ethylate dissolved in absolute alcohol and hydrogen chloride dissolved in absolute alcohol, we would have formed either a curdy precipitate, similar to the precipitate of silver chloride, or a stable colloidal system of a sodium chloride alcosol, depending upon the concentrations of the materials which were used. *The smaller the solubility of a substance in a solvent, the easier it is to prepare a colloidal system, and there is a maximum solubility above which a stable colloidal system, at least for lyophobic colloids, is impossible.* Taylor [1] has discussed this phase of the preparation of sols at greater length, and the reader is referred to him for a more elaborate treatment, cf. also Bradford.[2]

The concentrations of the solutions with which one works, determine in a large measure the nature of the system which will result. If one deals with concentrated solutions of very soluble substances which form a very insoluble precipitate, a colloidal gel is likely to result. Intermediate concentrations of the same materials may yield granular or crystalline precipitates which can be easily filtered off and which show no typical colloidal properties. Very dilute solutions of the same materials when mixed may yield an excellent colloidal sol. Figure 2 illustrates in a diagrammatic way the generalizations noted above.

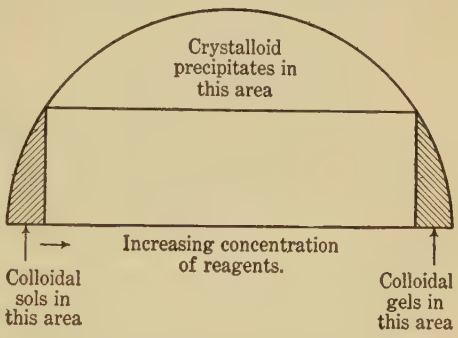

Crystalloid precipitates in this area
Increasing concentration of reagents.
Colloidal sols in this area
Colloidal gels in this area

FIG. 2.—A diagrammatic representation of the role of reagent concentration on the properties of the resulting systems.

Typical examples of the above generalization can be illustrated by laboratory experiments, using ferric chloride and potassium ferrocyanide to form Prussian blue. If one employs equimolecular concentrated solutions and mixes these solutions practically instantaneously, the resulting Prussian blue forms a dense gel, and if a small portion of this gel is stirred into a large volume of pure water, a stable colloidal sol of Prussian blue results. Such a sol will readily pass unchanged through a filter

[1] Taylor, W. W., The Chemistry of Colloids, Second Edition, pp. 171–179, Longmans, Green and Company, New York (1915).

[2] Bradford, S. C., Dispersoidology and the Theory of von Weimarn. Some Remarks upon the Significance of the Crystal and Colloid States, *Sci. Amer. Supplement*, No. 2255 : 182–183 (March 22, 1919).

paper. If now one dilutes these concentrated solutions eight or ten times and again mixes them, a precipitate of Prussian blue is formed which can be readily filtered off. If one further dilutes the initial solutions so as to have only a few milligrams of Prussian blue formed per 100 cc. of the resulting mixture, one obtains a stable sol of Prussian blue which again will readily pass through a filter paper. Accordingly if one wishes to filter off a precipitate of Prussian blue, the initial concentration of the reagents which are employed may be a determining factor in the complete retention of the precipitate on the filter. In some instances, such as in the colorimetric estimation of hydrocyanic acid, it is essential that all of the Prussian blue remain in the form of a colloidal sol. In such cases the initial concentration of the hydrocyanic acid solution must not exceed a certain value; the limit is probably near 4 milligrams of hydrocyanic acid per 100 cc. Under such conditions the Prussian blue sol will be stable, and its depth of color can be accurately measured in a colorimeter. If much greater amounts of hydrocyanic acid are present in the solution, a granular or crystalline precipitate is likely to form, and such a precipitate will settle rather readily and give low results when the depth of color is measured in the colorimeter.

Similar experiments can be conducted by using ferric chloride and sodium arsenite[3] or ferric chloride and sodium phosphate as the reacting materials. A series of beakers arranged with progressive dilutions of the reagents will show at one end of the series clear and apparently homogeneous gels and at the other end transparent, apparently homogeneous sols, while the intervening dilutions will show more or less complete precipitation grading in properties from one end of the series to the other. It is obvious that such phenomena are of extreme importance to the analytical chemist, and many of the instances where the precipitate "goes through the filter paper" can be avoided by choosing the proper concentration prior to making the precipitation.

A rather striking illustration of the influence of concentration of reagents is afforded by the data of Krienke.[4] The problem under consideration was to ascertain what effect the rate of addition of silver nitrate to an exactly equivalent solution of hydrochloric acid had on the occlusion of impurities in the resulting precipitate of silver chloride. The rate of addition of the silver nitrate was planned to be the only variable. As an indicator of the impurities carried down or adsorbed by the precipitate, use was made of a dilute solution of radium, inasmuch as

[3] Morrow, C. A., Biochemical Laboratory Methods for Students of the Biological Sciences. John Wiley & Sons, Inc., New York (1927).

[4] Krienke, O. K., "The Difference in the Amount of Radioactive Impurity Carried Down by Rapid or Slow Precipitation," unpublished manuscript.

extremely small quantities of radium can be determined with a very high degree of accuracy by the use of the electroscope. A known quantity of radium was added to the hydrochloric acid solution and then an exactly equivalent amount of silver nitrate added over various periods of time, the time of addition ranging from one second to four thousand seconds. Table I records certain of the data which Krienke obtained. When these data were plotted in the form of curves, the coordinates being time of addition of the silver nitrate solution and the radioactivity of the resulting precipitate or of the supernatant liquor, curves similar to those in Figs. 3 and 4 resulted. It will be noted that Fig. 4, showing the radioactivity of the mother liquor, is the reciprocal of Fig. 3, showing the radioactivity of the precipitate, silver

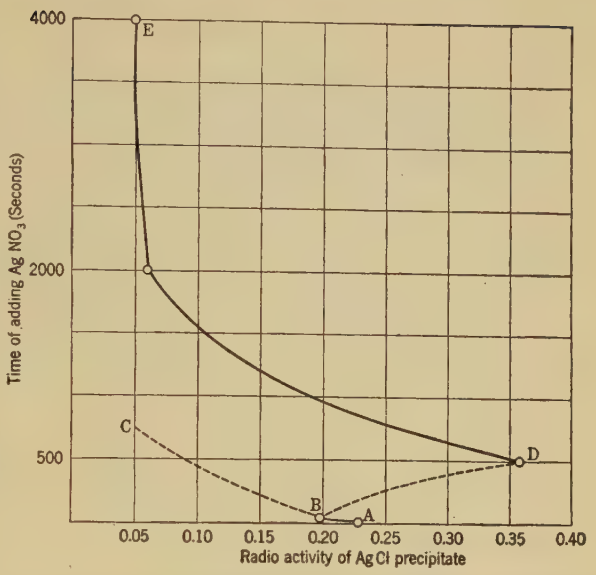


FIG. 3.—Showing the effect of the precipitating conditions on the occlusion of radioactive impurities in AgCl. Cf. Fig. 4. (Data of Krienke.)

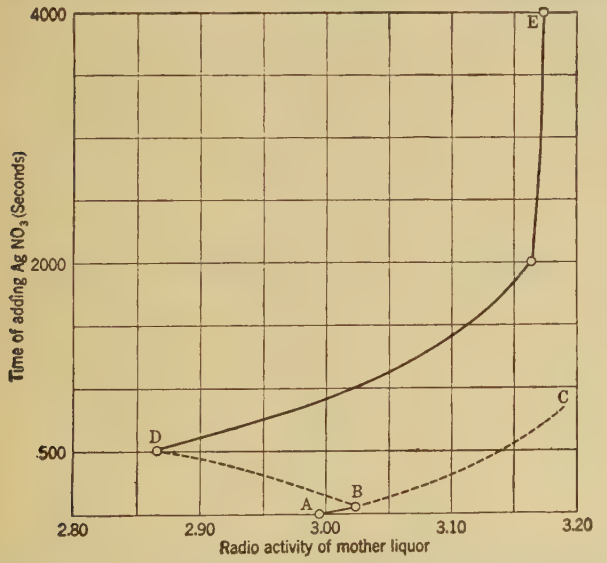


FIG. 4.—Showing the effect of the precipitating conditions on the occlusion of radioactive impurities in AgCl. Cf. Fig. 3. (Data of Krienke.)

TABLE I

A Study of the Effect of the Initial Concentration of $AgNO_3$ Solutions and the Time Rate of Addition of Such Solutions (Each Containing 0.8450 gm. of $AgNO_3$) to an Equivalent Solution of HCl, on the Occlusion of Impurities in the Resulting Silver Chloride Precipitate (Data of Krienke)

Experiment Number	Volume of Solution to which $AgNO_3$ Solution was Added	Solution Added (In Each Experiment 0.8450 gm. $AgNO_3$ was Added)	Time of Addition of $AgNO_3$ Solution
	Cubic Centimeters *	Cubic Centimeters	Seconds
1	195.0	5.0	1
2	195.0 †	5.0	20
3	144.5	55.5	500
4	144.5	55.5	2000
5	144.5	55.5	4000

Activity of Mother Liquor

Experiment Number	Series I	Series II	Series III
1	2.9556	2.9327	2.9943
2	2.9663	2.9449	3.0235
3	2.8380	2.8466	2.8647
4	3.0775	3.0673	3.1626
5	3.1067	3.0806	3.1728
Stand.	3.1912	3.1352	3.2222

Activity of Precipitate

(Activity of Standard Minus Activity of Mother Liquor)

Experiment Number	Series I	Series II	Series III
1	.2356	.2025	.2279
2	.2249	.1963	.1987
3	.3532	.2886	.3575
4	.1137	.0679	.0596
5	.0845	.0546	.0494

* Solutions 1 and 2 contained 5 cc. of standard radium solution and 5 cc. 1 N HCl, the remainder being water.

† Ten cc. of this 195 cc. added following addition of $AgNO_3$ (used to rinse beaker containing $AgNO_3$).

chloride. The solid lines, (*AB*) and (*DE*), represent the lines obtained from the experimental data, and the dotted line, (*BD*), is inserted to indicate how the curve might have been drawn, providing that the experiment had a single variable, *i.e.*, the time of the addition of the silver nitrate. The experimental curve then would be represented by (*ABDE*). It at first appeared as if experimental errors or some uncontrolled factor accounted for the abrupt change in form of the curve from (*AB*) to (*D*). However, a repetition of the above experiment invariably gave the same sort of data. A further study of the experimental conditions revealed the fact that Experiments 1 and 2 (the one-second and twenty-second times of additions) were performed by allowing the silver nitrate solution to flow from the tip of a 5 cc. pipette. It was impracticable to use a 5 cc. pipette to control the rate of flow for the longer periods, and accordingly a burette was employed, *the silver nitrate solution being diluted from a volume of* 5 *cc. to* 55.5 *cc.* with a correspondingly smaller amount of water being present in the hydrochloric acid solution to which the silver nitrate solution was added. The net effect of this change in experimental technic was to add a more dilute solution of silver nitrate to a more concentrated solution of hydrochloric acid, although there was no change in the actual weight of silver nitrate or hydrochloric acid which was used. The mere change in initial concentrations produced the appreciable change in the amount of radium carried down by the precipitate. Probably what actually happened was an alteration in the surface area of the resulting precipitated silver chloride, so that the precipitate formed on the addition of the more dilute silver nitrate had a greater surface area and consequently a greater adsorptive capacity. Referring to Fig. 2, the precipitating conditions were shifted from some point within the boundary of the crystalloidal field to a point nearer the boundary of the colloidal sols.

According to the above explanation, which admittedly involves assumptions which Krienke did not definitely prove, the dotted lines (*BD*) in Figs. 3 and 4 are without justification, and instead the dotted lines should have been extensions of the lines (*AB*) to some point approximately in the neighborhood of (*C*), and we would thus have two curves (*AC* and *DE*) more or less parallel to each other, representing the actual experimental conditions, (*AC*) representing an experiment in which 5 cc. of solution containing 0.8450 gram of silver nitrate was added, over varying periods of time, to 195 cc. of hydrochloric acid exactly equivalent to the silver nitrate which was added, and the curve (*DE*) representing the addition of 55.5 cc. of a solution containing the same amount of silver nitrate to 144.5 cc. of hydrochloric acid equivalent to the silver nitrate added. This series of experiments opens up an

entirely new field in analytical chemistry and indicates rather definitely the need of exact studies of the effect of concentration of reagents on the purity of the resulting precipitate and on the conditions which are necessary for complete precipitation and ease of filtration and washing of the precipitate.

Before we consider the various methods by which colloidal sols may be formed, it is necessary to note one additional property characteristic of stable colloidal systems. *The micelles present in colloidal systems possess either a positive or a negative electric charge.* The origin of this charge is still a matter of dispute, and in all probability the electric charge may arise from several causes such as the *direct ionization* of the material comprising the micelle, *the capture of an ion by the micelle* (adsorption, *vide infra*) in which case the micelle attracts to it an ion of an electrolyte and this, becoming fixed on the surface, gives rise to either a positive or a negative charge, or *electrification by contact* with the dispersions medium, in the same way that a wax rod becomes charged when rubbed with a woolen cloth.

Most hydrosols contain negatively charged micelles, whereas sols where turpentine is the dispersions medium ordinarily contain positively charged micelles. The explanation for the reversal of charge when we pass from water to turpentine has been ascribed to electrification by contact and has given rise to the general rule that *micelles are usually negative when in contact with a liquid having a high dielectric constant, and positive when in contact with a liquid having a low dielectric constant.* The dielectric constant of water is 81, which is very high as compared to most liquids. Hydrogen peroxide (46 per cent) is somewhat higher, 84.7. Certain of the dielectric constants of common liquids are shown in Table II.

TABLE II

Showing the Dielectric Constants of Certain Common Liquids

	Temperature, ° C.	Dielectric Constant		Temperature, ° C.	Dielectric Constant
Methyl alcohol	13.4	35.3	Carbon disulfide	17.0	2.64
Ethyl alcohol	14.7	26.8	Carbon tetrachloride	18.0	2.25
Glycerine	15.0	56.2	Benzene	19.0	2.26
Acetone	20.0	21.5	Turpentine	20.0	2.23
Acetic acid	18.0	9.7	Petroleum	21.0	2.12
Chloroform	18.0	5.2	Olive oil	21.0	3.11
Ethyl ether	18.0	4.368	Phenol	48.0	9.68

As a general rule only the oxides and hydroxides of metals and basic organic compounds are positively charged in water.[5] In these instances we are probably dealing with either the phenomena of ionization or of the adsorption of an ion. Thus, silver hydroxide or ferric hydroxide might be regarded as ionizing according to the following scheme:

$$AgOH \rightarrow Ag^+ \text{ and } OH^-$$

$$[Fe(OH)_3]_x \rightarrow [Fe(OH)_2]_x^+ \text{ and } OH^-$$

Or we might postulate that the ferric hydroxide micelle still contained a small amount of ferric chloride, in which case we could have an ionization as follows:

$$[Fe(OH)_3]_x(FeCl_3)_y \rightarrow [Fe(OH)_3]_x(FeCl_2)_y^+ \text{ and } Cl^-$$

It will be noted that in each one of these instances we have a negative ion given off into the solution with a corresponding residual positive charge on the micelle. Other discussions of the electrical charge will be given in greater detail in the following pages.

PRACTICAL METHODS FOR THE PREPARATION OF COLLOIDAL SYSTEMS

As already noted at the beginning of this chapter, the methods of preparation may be divided into (A) Crystallization or Condensation Methods, where we pass from a true solution to colloidal systems; or (B) Solution or Dispersion Methods, where we pass from matter in mass to colloidal micelles.

A. Crystallization or Condensation Methods.—These methods may be divided into six general groups.

1. The Cooling of a Liquid.—Typical examples are water in ether, cooled by carbon dioxide snow, giving rise to a colloidal sol of ice-ether. Referring to Formulae (1) and (2), we can see that the lowering of the temperature has (a) reduced the solubility of the water in the ether, so that the supersaturation $(C - L)$ is much greater, and likewise (b) it has reduced the diffusion coefficient (Δ) of the Noyes-Nernst formula and increased the viscosity which will have the same effect as increasing the length of the diffusion path (l). We have thus made for the formation of a greater number of nuclei and have a much slower rate of crystal growth. Accordingly an ice-ether sol is relatively stable at a very low temperature. Other examples similar to the above could be cited with the same general argument.

[5] There are some exceptions to this, but as a generalization it is fairly satisfactory.

2. Replacement of Solvent.—This is a very common method for the formation of colloidal systems, and the success of the method depends again upon factors noted in Formulae (1) and (2). If we have two solvents, one more or less soluble in the other, one liquid being an excellent solvent for some material which is at the same time practically insoluble in the other liquid, we have conditions where the replacement-of-solvent method can be employed. Thus, for example, sulfur is relatively soluble in carbon disulfide but extremely insoluble in water. If a few drops of a solution of sulfur in carbon disulfide is rapidly stirred into a large volume of water, a beautiful hydrosol of sulfur results. We have here suddenly decreased (L) and increased the initial values of $(C - L)$, so that many nuclei are formed. The final value of (L) is so extremely low that almost instantaneously the initial supersaturation $(C - L)$ falls to practically zero, due to the formation of nuclei. Accordingly when crystal growth takes place $(C - L)$ is essentially zero and therefore the sol is stable for a long period of time.

Obviously a sulfur hydrosol is not of any great importance to the biologist or to the biological chemist. However, anyone working with biological preparations will very soon experience the phenomena which are characteristic of this method of the preparation of colloidal systems.

A very common method for the purification of such materials as gums, proteins, enzymes, etc., is to take advantage of their insolubility in alcohol in order to precipitate them in a form which can be filtered. If one is dealing with a water-soluble protein, it is possible to concentrate it by vacuum evaporation down to a rather viscous sol. If one pours this sol into absolute alcohol, the protein is, as a rule, precipitated in the form of flocs which can be readily gathered and in many instances even filtered off. As the purification progresses, however, and the above process of alcohol precipitation is repeated several times, it frequently happens that no precipitate is formed when the aqueous sol is poured into the alcohol, but instead we have only a limpid, opalescent sol resulting. The problem then arises as to how such a sol can be "broken" so as to cause the formation of the desired precipitate. This can usually be effected by the addition of a small amount of electrolyte (*vide infra*), such as a drop of saturated sodium chloride or preferably lithium chloride. In some instances sols of this sort are very stable and require a considerable amount of experimentation before the dispersed phase is precipitated.

The beginner in biological preparations is probably less likely to lose valuable material when a complete sol results, in an attempted alcohol precipitation, than he is when only a partial sol is formed. It very often happens that a part of the material precipitates in the expected

manner, while a greater or a smaller portion remains in the form of a slightly opalescent alcosol. Under such conditions the worker is very likely to decant and discard the supernatant liquor, retaining only the precipitated portion. Many instances have been known where 90 per cent of valuable material went down the drain, due to the decantation of supernatant sols. In the preparation of any material by precipitation by pouring into another solvent, it is highly desirable to check the solid content of the supernatant liquor in a qualitative fashion before the supernatant liquor is discarded, in order to be sure that a part of the desired material is not suspended in the form of colloidal micelles.

3. Reduction Methods.—These methods depend upon the reduction of a soluble metallic salt to the insoluble metal. In this group of methods in particular the purity of the water is all-important. Distilled water which has stood for several hours in an ordinary soft-glass bottle usually contains sufficient electrolytes to render the production of the desired sol uncertain. Likewise the purity of the reagents that are employed becomes a very important factor. Typical examples of colloidal systems formed by the reduction methods are the reduction of auric chloride by formaldehyde, phosphorus, phenylhydrazine, or tannin, as noted by Morrow[6] or by Holmes.[7] Similarly a beautiful silver sol can be prepared by reduction with hydrogen. If a stream of hydrogen is passed into a hot solution of freshly precipitated silver hydroxide, reduction takes place, and an intensely yellow, essentially electrolyte-free sol of silver results. The reaction which takes place can be expressed as

$$2AgOH + H_2 \rightarrow 2Ag + 2H_2O.$$

The sols that are formed by reduction methods are, as a rule, very sensitive to the action of electrolytes and form, from the standpoint of laboratory experimentation, one of the most interesting groups of colloidal systems.

Colloidal metals are gradually coming into use in medicine. Recently colloidal lead has been suggested as a suitable therapeutic agent in the treatment of cancer, and reduction methods appear to be the most suitable for the production of such metallic sols.

4. Oxidation Methods.—Relatively few examples of colloidal systems formed by the use of oxidation processes are recorded. The outstanding example perhaps is the oxidation of hydrogen sulfide and the

[6] Morrow, C. A., Biochemical Laboratory Methods for Students of the Biological Sciences, pp. 12, 13, and 14.

[7] Holmes, H. N., Laboratory Manual of Colloid Chemistry, pp. 31 and 32. John Wiley & Sons, Inc., New York (1928).

resulting formation of a sulfur hydrosol, according to the following scheme:

$$H_2S + O_2 = 2S + H_2O.$$

This sol should be familiar to everyone who has ever had any laboratory chemistry. Those who are not interested in colloid phenomena are usually exasperated when they return to the laboratory and find that their solution of hydrogen sulfide has turned to a milky-appearing liquid, and the active hydrogen sulfide has disappeared from the solution. If the bottle in which the hydrogen sulfide solution was stored had been completely filled with the solution and tightly stoppered, this oxidation would not have occurred, but under ordinary laboratory conditions it is an inevitable occurrence. A later stage is the coagulation of the sulfur hydrosol and the precipitation of crystalline sulfur in the bottom of the bottle. Precipitation is, however, rarely complete, and the supernatant liquid over such a sulfur precipitate is usually a dilute sulfur hydrosol. Selenium and tellurium form sols similar to the sulfur hydrosols.

5. Hydrolysis Methods.—Many materials which are readily soluble in water can be made to undergo hydrolysis with the production of an extremely insoluble residue. In such instances appropriate technic will result in the formation of stable sols. Ferric chloride may be chosen as possibly the best example of such a material. The reaction may be written as

$$FeCl_3 + 3H_2O = Fe(OH)_3 + 3HCl.$$

Experiments based on this reaction are given by Morrow[8] and by Holmes.[9] As a matter of fact the above reaction is some what misleading in that it indicates the formation of micelles composed entirely of ferric hydroxide. As a rule the micelles are not pure ferric hydroxide, but rather are a mixture of ferric hydroxide and ferric chloride in varying ratios. Taylor[10] discusses this phenomenon under the title, "The 'Complex' Theory of Colloids," and points out that pure ferric hydroxide sols, or as a matter of fact most of the lyophobic sols, are very unstable when they are entirely free from admixture with stabilizing ions. The micelles in the ferric hydroxide sols as ordinarily prepared are undoubtedly represented by the formula, $[Fe(OH)_3]_x(FeCl_3)_y$, and probably all have a positive charge, due to the ionization of the residual ferric chlo-

[8] Morrow, C. A., Biochemical Laboratory Methods for Students of the Biological Sciences, pp. 15 and 16.

[9] Holmes, H. N., Laboratory Manual of Colloid Chemistry, pp. 34 and 35.

[10] Taylor, W. W., The Chemistry of Colloids, p. 108. Longmans, Green and Co., New York (1915).

ide as noted above. Browne[11] has made studies of the hydrogen-ion concentration and the chloride-ion concentration in such sols and finds that the chloride-ion concentration is more or less proportional to the stability of the sol, indicating that ferric chloride is adsorbed on the ferric hydroxide micelles. Recently Sorum[12] has prepared for the first time a ferric hydroxide sol which is chloride-free.

The hydrolysis methods are applicable to the formation of many hydrosols of the metallic oxides and hydroxides.

6. Double Decomposition or Precipitation Methods.—In many instances the product of the interaction of two soluble materials is an extremely insoluble precipitate. Under such conditions by proper manipulation the precipitate can usually be obtained in the form of a sol. Typical examples that one meets with in the analytical laboratory are hydrosols of barium sulfate or arsenous sulfide as noted in the following reactions:

$$BaCl_2 + H_2SO_4 = BaSO_4 + 2HCl$$

$$As_2O_3 + 3H_2S = As_2S_3 + 3H_2O.$$

Probably every analytical chemist has had the undesirable experience of a barium sulfate precipitate going through the filter paper. The conditions of precipitation had been such that a more or less stable hydrosol had been formed. Such conditions are usually brought about when there is a nearly exact equivalence of barium chloride and sulfuric acid and when the resulting concentration of hydrochloric acid is very low. Precipitation at the boiling temperature and the general practice of allowing a barium sulfate precipitate to stand for a number of hours before filtering, both favor the growth of crystals and the subsequent increase in particle size, so that the precipitate is retained on the filter paper. Following the formation of the nuclei, the elevated temperature increases (L) the solubility of the disperse phase and ($C - L$) the absolute supersaturation, at the same time increasing (Δ) the diffusion coefficient and decreasing viscosity which will likewise increase the diffusion coefficient. The length of the diffusion path (l) will be decreased, due to the convection currents and stirring at the higher temperature, so that it may become essentially negligible.

Recently Trimble[13] has pointed out that crystal growth ceases to

[11] Browne, F. L., The Constitution of Ferric Oxide Hydrosol from Measurements of the Chlorine- and Hydrogen-ion Activities, *J. Am. Chem. Soc.*, 45 : 297–311 (1923).

[12] Sorum, C. H., The Preparation of Chloride-free Colloidal Ferric Oxide from Ferric Chloride, *J. Am. Chem. Soc.*, 50 : 1263–1267 (1928).

[13] Trimble, H. M., The Coalescence of an Unfilterable Precipitate of Barium Sulfate, *J. Phys. Chem.*, 31 : 601–606 (1927).

be a factor when the particles of barium sulfate are larger than two microns apparent diameter, but that aggregation and cementing together of the particles is an important factor in the retention of the precipitate on the filter paper.

In the arsenous sulfide sols there is no precipitating ion formed by the reaction, and extremely stable sols can accordingly be prepared. Here again we are probably not dealing with pure As_2S_3 micelles, but probably we have again a complex ion of arsenous sulfide stabilized by the sulfhydril (SH^-) ion, due to the presence of a slight excess of hydrogen sulfide, so that the micelle actually possesses the formula,

$$(As_2S_3)_xSH^-.$$

It is a common experience for the analytical chemist in precipitating the sulfides of arsenic, antimony, tin, silver, etc., to find that they occasionally are not retained on a filter paper. In such instances hydrogen sulfide has been passed into the solution for too long a period of time and the initial precipitate of sulfide has become stabilized by the adsorption of some of the excess hydrogen sulfide with a resulting hydrosol formation.

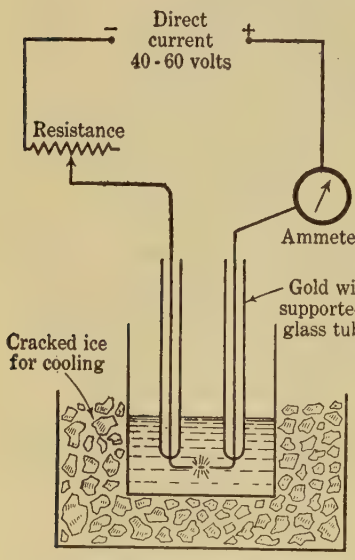


FIG. 5.—A diagrammatic illustration of the Bredig method of preparing metallic sols by electrical pulverization.

B. SOLUTION OR DISPERSION METHODS.—These methods may be divided into two general groups: (1) electrical dispersion, and (2) peptization.

1. Electrical Dispersion. — Bredig,[14] in 1898, announced a general method for the preparation of metallic sols by the process of electrical pulverization. It is well known that when an arc is passed between two electrodes, one of the electrodes becomes corroded. Bredig made use of this phenomenon by striking an arc between metallic electrodes inserted under the surface of a liquid. Figure 5 is a diagrammatic representation of such an apparatus. Using thick wires of gold, platinum, silver, etc., and a direct current of 4 to 10 amperes at 35 to 50 volts, he was able to prepare metallic sols

[14] Bredig, G., Darstellung colloidaler Metallösungen durch elektrische Zerstäubung Z. *angew. Chem.*, 1898, p. 951–954.

not only in water as a dispersions medium but also in certain of the organic solvents, although the latter offer difficulties, owing to the separation of colloidal carbon by the decomposition of the organic liquid in the heat of the electric arc.

When the points of two gold wires are momentarily brought together beneath the surface of the water and then separated, an arc is struck between the two points. The tip of the cathode becomes molten in the intense heat of the arc and a stream of fine gold particles is shot from the molten cathode toward the anode. In the heat of the arc and under the influence of the electric current some of the smaller of these particles may vaporize or become re-subdivided; in either event part of the gold fails to reach the anode, instead being dispersed in a colloidal cloud which rises through the liquid from the vicinity of the arc with the appearance of a rising cloud of smoke or dust. The gold particles which do reach the anode fuse on its tip, so that the wire forming the anode lengthens as the wire forming the cathode shortens. A certain amount of coarse, granular gold dust, derived from larger particles of gold which failed to reach the anode, is deposited in the bottom of the beaker. It is preferable to bring the electrodes in contact and later to separate them so as to strike the arc, using a mechanical device of springs and set screws, rather than to attempt to operate the electrodes by hand. Kraemer and Svedberg[15] have modified Bredig's original method, making use of a high-frequency alternating current arc instead of a direct current, and by use of this modified technic have been able to prepare metallic organosols containing a minimal amount of carbon due to organic decomposition. Svedberg[16, 17] had earlier made use of an induction coil for the preparation of similar sols.

It is obvious, from a consideration of the facts that both vaporized metal and finely divided molten metal contribute to the Bredig sols, that such sols cannot be expected to be uniform with regard to particle size. In general, such sols contain a rather wide range of particle size, the sizes varying from particles which gradually settle out, down to particles near the lower limit of the colloid realm. The range in particle size in sols obtained by the Bredig method is much greater than it is in metallic sols obtained by the reduction methods, and as a general rule it is easier to obtain sols with small-sized particles (10–25$m\mu$) using reduction methods,

[15] Kraemer, E. O., and Svedberg, The, Formation of Colloid Solutions by Electrical Pulverization in the High-Frequency Alternating Current Arc, *J. Am. Chem. Soc.*, 46 : 1980–1991 (1924).

[16] Svedberg, The, Ueber die elektrische Darstellung einiger neuen colloidalen Metalle, *Ber.*, 38 : 3616–3620 (1905).

[17] Svedberg, The, Ueber die elektrische Darstellung colloidaler Lösungen, *Ber.*, 39 : 1705–1714 (1906).

than it is to obtain similar sols by the Bredig method. The noble metals rather readily yield sols by the Bredig method. Using other metals as electrodes the sols which are obtained are usually contaminated with larger or smaller amounts of oxides and hydroxides.

Bredig's method offers an extremely valuable technic for fundamental studies in the properties of colloid systems, due to the fact that sols can be obtained by striking an arc between pure metallic electrodes under conductivity water, the sols therefore containing only metallic particles suspended in a pure liquid. In all probability sols of greater purity can be obtained by the Bredig method than by any other method of preparation. They can be obtained in the entire absence of electrolytes, and as a result the influence of minute traces of electrolytes on colloid stability has been extensively investigated, using Bredig sols as the colloid system under study. Burton[18] has made extensive observations of the behavior of metallic sols, particularly silver sols prepared by Bredig's method. For a more extended discussion of the Bredig sols, the reader is referred to Freundlich[19] and Svedberg.[20]

2. Peptization.—Peptization is undoubtedly the most important of all dispersion methods. It appears to be a general rule that a peptizer, the substance which causes the matter in mass to disperse, either (*a*) must have one ion in common with the material to be dispersed, or (*b*) must be capable of forming a soluble compound with the material to be dispersed, or (*c*) must have one ion which is very strongly adsorbed by the material being dispersed. In general the peptizer acts chemically on the insoluble material, so that peptization may be regarded as at least in part a truly chemical reaction, although in some instances it is difficult to write a stoichiometrical equation. Adsorption probably plays a major role in peptization. We have already noted the "complex theory of colloids," using the example of a ferric hydroxide sol where the micelle probably has the formula of $[Fe(OH)_3]_x(FeCl_3)_y^+$. In this case the ferric chloride can be regarded as the peptizing agent of the ferric hydroxide. If precipitated ferric hydroxide is suspended in water and heated to near the boiling point (the heat is merely to hasten the reaction) and a small amount of hydrochloric acid added, it will be found that within a short time the precipitated flocculent ferric hydroxide has dispersed to a transparent yellow-brown sol which will readily

[18] Burton, E. F., On the Properties of Electrically Prepared Colloidal Solutions, *Phil. Mag.*, (6) 11 : 425–447 (1906).

[19] Freundlich, Herbert, Colloid and Capillary Chemistry, pp. 509–513. Translated from the German by H. S. Hatfield. E. P. Dutton and Company, New York (1926).

[20] Svedberg, The, Colloid Chemistry, Second Edition, The Chemical Catalog Company, New York (1928).

pass through a filter paper. The amount of hydrochloric acid which is necessary to bring about such a peptization may be less than 1 per cent of the amount which would be necessary to convert into ferric chloride the ferric hydroxide which is present. The small amount of ferric chloride which is formed by this reaction acts as a peptizing agent on the flocculent ferric hydroxide, dispersing the flocs into smaller aggregates of colloidal dimensions and stabilizing these aggregates probably by adsorption of a small amount of the ferric chloride.

A similar sol can be made by the peptization of aluminium hydroxide with hydrochloric acid.[21] Peptization can likewise be induced by adding ferric chloride to ferric hydroxide[22] or aluminium chloride to aluminium hydroxide. Even ammonium hydroxide may act as a peptizing agent, and it is for this reason that texts in analytical chemistry stress the fact that the ammonia used in precipitating the R_2O_3 group of metals must be boiled off before the precipitate is filtered. Otherwise a certain amount of aluminium hydroxide sol may pass through the filter paper, and accordingly a loss of aluminium would result.

In the case of sulfides we have a very efficient peptizing agent in hydrogen sulfide. This has already been noted in the case of the arsenous sulfide sols where the reaction probably is

$$As_2S_3 + H_2S \rightarrow (As_2S_3)SH^-.$$

It should be stressed that such micelles as

$$(As_2S_3)SH^-,\ [Fe(OH)_3]_xFe^{+++},\quad \text{or}\quad [Fe(OH)_3]_x(FeCl_2)_y{}^+,\ \text{etc.},$$

are not chemical compounds in the sense that they represent a pure chemical individual. Their composition is not constant but will vary continuously through a wide range, depending on the amount of peptizing agent which is adsorbed.

The analytical chemist who ignores the phenomena of peptization is very likely to secure results which are seriously in error, for many of the precipitates of gravimetric analyses are readily peptized when in a moist condition. The silver salts, silver chloride, silver bromide, and silver iodide may be cited as common examples. Figure 6 illustrates diagrammatically some general phenomena applicable to the gravimetric determination of silver as silver bromide and to the peptization of precipitated silver bromide to form stable silver bromide sols. If exactly equivalent solutions of silver nitrate and potassium bromide are mixed, an isoelectric precipitate of silver bromide will be formed, as shown at the point

[21] Morrow, C. A., Biochemical Laboratory Methods for Students of the Biological Sciences, p. 18.

[22] Holmes, H. N., Laboratory Manual of Colloid Chemistry, p. 41.

(*C*) on the diagram. *This is the only point on the diagram where strictly pure silver bromide is found and where the weight of the precipitate accurately represents the amount of silver present in the solution.* The precipitates which are obtained at the points (*A*) and (*E*) will be heavier than the precipitate obtained at (*C*), and accordingly will give high results for bromine and silver respectively, whereas precipitates obtained anywhere along the curve (*AC*) will be light in weight and give low results for bromine, or along the curve (*CE*) will give low results for silver. Anyone who is interested in a consideration of the nicety of technic necessary for the *accurate* determination of silver should read some paper dealing with the determination of atomic weights where silver is used as the reference standard. It is in such determinations that extreme accuracy

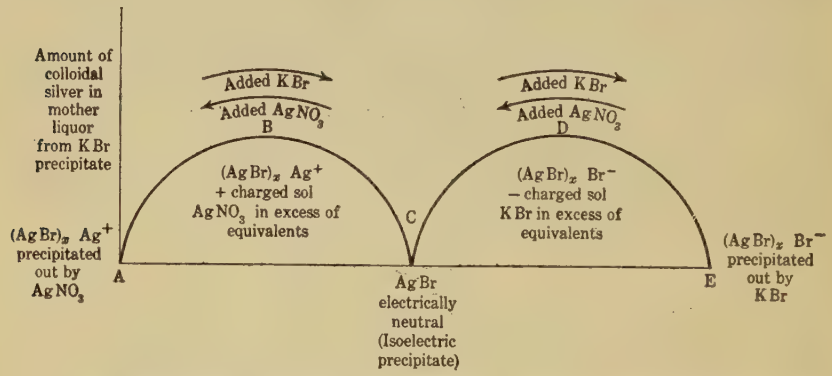

Amount of colloidal silver in mother liquor from KBr precipitate
Added KBr
Added AgNO3
B
(AgBr)x Ag+ + charged sol AgNO3 in excess of equivalents
(AgBr)x Ag+ precipitated out by AgNO3
A
C
AgBr electrically neutral (Isoelectric precipitate)
Added KBr
Added AgNO3
D
(AgBr)x Br− − charged sol KBr in excess of equivalents
(AgBr)x Br− precipitated out by KBr
E

FIG. 6.—A diagrammatic illustration of peptization, the influence of ions on the sign of the charge on the micelle, isoelectric precipitation, and "salting out" at high salt concentrations.

is necessary, and in such experiments the precipitation of isoelectric silver bromide or chloride is rigidly controlled.

Referring to the diagram it will be seen that if one adds an excess of potassium bromide to the precipitated silver bromide, the amount of silver in the mother liquor follows the curve (*CDE*), the point reached depending on the amount of potassium bromide added in excess. Throughout the entire area under the curve (*CDE*) there is formed a precipitate of silver bromide containing adsorbed bromine ions and the supernatant liquid is a negatively charged silver bromide sol, probably of the composition, $(AgBr)_xBr^-$. At point (*D*) on the curve we attain the greatest concentration of the negatively charged silver bromide micelles, a further addition of potassium bromide causing precipitation of the colloidal particles, so that finally at (*E*) there is no silver in the mother liquor and we have a precipitate which appears to be silver bro-

mide but in reality is silver bromide containing a considerable excess of bromine and probably likewise contaminated with potassium bromide.

A similar argument can be made for the region lying under curve (*ABC*) except that in this instance silver nitrate is added in excess. Throughout this area we have a positively charged sol in the mother liquor, the composition of the micelles being represented as $(AgBr)_zAg^+$. This sol reaches its maximum concentration at point (*B*) and is precipitated by the excess silver nitrate at point (*A*), the precipitate having the composition noted on the diagram. The above example has been cited in considerable detail merely because it is typical of the behavior of many precipitates met with in the various fields of chemistry. The same general argument will apply in numerous instances.

The peptization of matter in mass may be brought about in many ways, chemically, mechanically, and electrically. In each instance, however, *energy must be added to the system.* As we shall see when we consider surface phenomena (*vide infra*), a given mass of material dispersed in small micelles has a much greater energy content than the same mass of material when present in large aggregates. This energy must be added to the system in some form if we are to peptize matter in mass.

Peptizing methods fall into four general classes.

a. The Washing Out of a Precipitating Agent.—Everyone familiar with quantitative analysis recognizes the fact that certain precipitates cannot be washed with pure water without causing the precipitate to go through the filter paper. Thus, for example, we wash the "yellow precipitate" of ammonium phosphomolybdate with a dilute solution of ammonium nitrate rather than with pure water. The ammonium nitrate prevents the precipitated ammonium phosphomolybdate from dispersing into a colloidal sol. Similarly, if we were to wash the precipitate of silver bromide obtained at points (*A*), (*C*), (*E*) on the diagram in Fig. 5, we would find that the curves (*ABC*) and (*CDE*) were reversible and that as the silver nitrate or potassium bromide was washed out of precipitates (*A*) and (*E*), the liquid passing through the filter would contain silver bromide micelles positively charged in the case of precipitate (*A*) and negatively charged in the case of precipitate (*E*). The isoelectric precipitate obtained at point (*C*) would not be peptized by the action of water, and *such isoelectric material is the only material which would give accurate information in regard to the solubility of silver bromide in water.* In the great majority of instances where a precipitate goes through the filter paper we can be sure that we are dealing with the peptization of a precipitated colloid system (a gel or a coagulum) by the washing out of the precipitating agent.

In the mechanical analysis of soils this phenomenon comes into play. If a soil is leached with hydrochloric acid and then washed free of chlorides with distilled water, it is the general experience that before the chlorides are completely removed clay begins to pass through the filter paper. The clay in the soil is usually present in a more or less precipitated condition, due to the presence of inorganic salts. The removal of these salts permits the peptization of the clay aggregates to take place with the formation of a clay hydrosol. If the soil be a surface soil, the black humus likewise becomes peptized by washing out the calcium or other metallic salts which hold it in a precipitated condition, and it often happens that appreciable amounts of humus can be extracted from the soil in the form of a colloidal sol by first leaching the soil with hydrochloric acid to remove the calcium, followed by washing with distilled water to the absence of chlorides.

Many organic substances are peptized by the direct addition of water. Familiar examples are gelatin, gum arabic, dextrin, etc. In certain instances it is necessary to add energy in the form of heat to cause complete dispersion. Thus, for example, agar and gelatin will form gels in cold water, but sols in hot water.

b. The Addition of a Peptizing Agent.—This has already been discussed fully in the sections above. Specific examples which could be cited are: the addition of ammonium hydroxide to a soil in order to form a clay hydrosol; the peptization of china clays by alkalies or carbon dioxide; the removing of dirt from clothing by soap; stable sols of lamp black and graphite by the use of soaps, proteins, gums, and resins; emulsions, such as kerosene emulsion, so widely used in spraying, by the use of soaps. A great number of laboratory or technical applications have been made [23] involving peptization of one or another material by the addition of a peptizing agent.

c. Mechanical Disintegration.—As noted above, peptization occurs whenever energy is added to a system. This energy may be added in the form of mechanical energy, *i.e.*, grinding. If one places a fragment of a microscope cover-glass in a mortar and retains sufficient enthusiasm to grind this fragment vigorously for twenty to thirty minutes, it will be found that a considerable part of the glass will remain as a stable hydrosol when water is added to the ground mass. Similarly, almost any practically insoluble material can be obtained in the form of a colloidal

[23] Compare, for example, Bancroft, W. D., Report on Peptization and Precipitation, Second Report on Colloid Chemistry, Brit. Assoc. for the Advancement of Science (1918); Bogue, R. H., The Theory and Application of Colloidal Behavior, Vol. II, McGraw–Hill Book Company, New York (1924); and Fall, P. H., Detergent Action of Soaps, *J. Phys. Chem.*, 31 : 801–849 (1927).

sol. Most insoluble materials form lyophobic sols. The formation of lyophobic sols requires a much greater expenditure of mechanical energy than does the formation of similar lyophilic sols. The great majority of lyophilic sols will form more or less spontaneously on the addition of the liquid.

Colloid mills have been devised to prepare finely divided materials for industrial uses. Most of these mills are misnamed "colloid" mills, inasmuch as the size of the suspended particle rarely falls within the limits fixed by the colloid realm. On the other hand, the disintegration produced by such mills is greater than that generally produced by other grinding methods, and such apparatus is coming more and more into use for the grinding of pigments, the making of emulsions or finely dispersing any desired material in a liquid.

d. Electrical Energy.—The formation of colloidal systems by the use of electrical energy may be regarded as a form of peptization. We have already discussed it under electrical dispersion, and it is simply referred to again at this point, inasmuch as some authors make no distinction between electrical dispersion and peptization. Whether or not such a distinction is made will depend upon how closely lines are drawn in a definition.

Emulsions.—Possibly emulsions could be classified as a fifth group of colloidal systems formed by peptization. However, as already noted (p. 11) the range of particle size in emulsions generally exceeds the upper limit of the colloid realm, so that many of the fat droplets in emulsions are visible in the ordinary microscope field.

An emulsion, in its simplest form, can be defined as a mixture of two mutually insoluble liquids. It is obvious from this definition that *two different types of emulsions are possible, depending upon which liquid forms the disperse phase and which liquid forms the dispersions medium.* When water is one of the components we distinguish these two forms of emulsions by oil-in-water or water-in-oil emulsions. Emulsions which are met with in biological phenomena almost invariably have water as one of the phases.

It is extremely difficult to produce simple emulsions, *i.e.*, emulsions containing only one pure liquid dispersed in another pure liquid, which have any appreciable concentration and which are stable. In certain instances dilute emulsions can be obtained where the above conditions are met. For example, in the preparation of aniline by steam distillation, the supernatant liquid in the receiving flask very often is a milky appearing liquid, a dilute emulsion of aniline in water. Such emulsions are often obtained in the distillation of volatile liquids which are relatively insoluble in water. If the bulk of such emulsions is small, this

phenomenon does not constitute a serious loss in industrial processes unless the suspended material is extremely valuable as is the case, for example, when one is dealing with certain essential oils, in which case the material remaining in the emulsion must be recovered either by extracting with a solvent or by breaking the emulsion in some manner, causing the oil to separate.

Most emulsions used in the industries are stabilized by the presence of another disperse system, preferably a colloid which shows a marked affinity for either the oil or the water. Gums are the substances most generally used as stabilizers. Gum acacia (gum arabic) is the common stabilizer for oil-in-water emulsions, although proteins act similarly. Probably the reason that the emulsions used in the industries are stabilized with gum acacia instead of proteins, is due to the fact that proteins are readily attacked by putrefactive bacteria, whereas gum acacia is not. Gum acacia disperses in water to form a lyophilic hydrosol. It is a general rule that *those emulsion stabilizers which are soluble in water or which form lyophilic hydrosols produce emulsions of the oil-in-water type, whereas those stabilizers which are insoluble in water and soluble in oil produce emulsions of the water-in-oil type.* The sodium or potassium soaps, being water-soluble, act as stabilizers of oil-in-water emulsions, whereas the calcium soaps, being water-insoluble and oil-soluble, serve as efficient stabilizers for water-in-oil emulsions. Holmes and Cameron[24] have pointed out that gum dammar, an oil-soluble gum, is an efficient stabilizer of water-in-oil emulsions.

As a rule the natural emulsions, such as milk and the milky latex of plants, are stabilized by proteins. Thus the rubber hydrocarbon is held in the latex by a protein which has more or less the characteristics of a globulin. The proteins (and those phosphatides and lipides which are fairly efficient hydrophilic colloids) stabilize the fat droplets in milk and cream. The egg yolk proteins stabilize mayonnaise dressing, and a little gelatin or better still gum dammar will make a very stable French dressing emulsion from olive oil and vinegar.

There is apparently no limit to the amount of the disperse phase which may be present in an emulsion. Pickering[25, 26] has pointed out that 99 *per cent of oil can be dispersed in* 1 *per cent of water containing an emulsifying agent to form an oil-in-water emulsion.* The liquid droplets apparently pack and deform into a more or less honeycomb structure with only a thin film of water between them. Obviously such emulsions

[24] Holmes, H. N., and Cameron, D. H., Gum Dammar as an Emulsifying Agent, *Science*, 56, 724 (1922); Emulsion, U. S., Patent 1,429,430, dated Sept. 19, 1922.

[25] Pickering, S. U., Ueber Emulsionen, *Koll. Z.*, 7 : 11–16 (1910).

[26] Pickering, S. U., Emulsions, *J. Chem. Soc., London*, 91 : 2001–2021 (1907).

possess a certain degree of rigidity or a rather high viscosity. It is doubtful if such concentrated emulsions could be formed without a distortion of the fat droplets, for a mixture of such concentration could not be obtained in the case of a solid dispersed in water. For example, lead

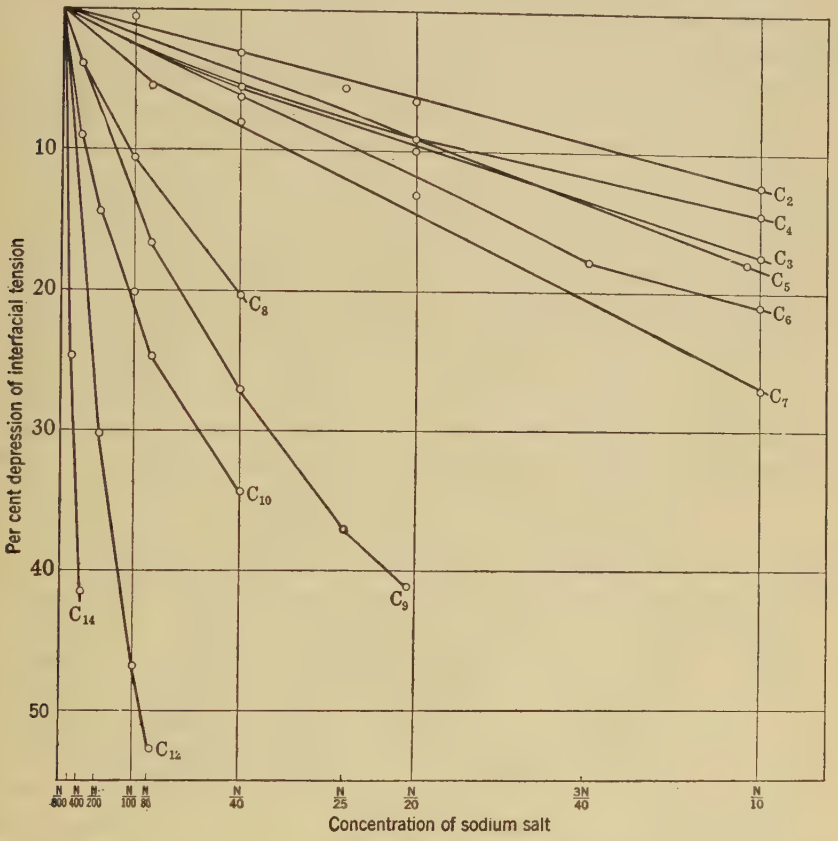


FIG. 7.—The effect of the sodium salts of homologous series of fatty acids on the lowering of the interfacial tension of a water-paraffin oil system. (Data of Donnan and Potts.)

shot and water reach a limit of shot content depending on the size of the shot.

Pickering likewise points out that various solid materials, such as barium sulfate, precipitated sulfur, Prussian blue, Trumbull's blue, and purple of Cassius can be used to stabilize emulsions. In such instances the precipitate has an affinity for either the water or the oil and collects on the surface of the droplet, forming a more or less rigid membrane

which prevents a later coalescence of the disperse phase. Schlaepfer[27] concluded that a finely divided solid which was more easily wetted by oil than by water should be capable of forming emulsions with water as a disperse phase. He used lamp black in order to test out his theory and formed stable water-in-oil emulsions. Hatschek[28] has given an excellent discussion, with bibliography, of emulsion formation and stability. Likewise Fischer and Hooker[29] have discussed the general theory and certain relationships of emulsion formation to pathological conditions.

Emulsions are stable when the interfacial tension between the phases is low, and *any substance which greatly lowers the interfacial tension of water will tend to stabilize an emulsion of which water is one component.* Donnan and Potts[30] have shown that the first fatty acid to produce an emulsifying effect is lauric acid (C_{12}), and they have likewise shown that this is the first one to greatly lower the surface tension of water at low concentrations of the sodium salt. Table III, calculated from the data of Donnan and Potts, shows the percentage lowering of the interfacial tension between pure paraffin oil and water, when relatively small quantities of the sodium salts of a homologous series of the saturated aliphatic acids are added to the aqueous phase, and Fig. 7 is a graphic representation of the experimental data of Donnan and Potts.

Finkle, Draper, and Hildebrand,[31] and Harkins[32] are undoubtedly correct in ascribing the action of efficient emulsifiers to the collecting of the emulsifying agent in the interfacial film in such a manner that the oil droplets are surrounded by a " shell " of oriented molecules of the stabilizing agent. The theory which Finkle, Draper, and Hildebrand propose to explain the formation of oil-in-water emulsions when the alkali salts of the fatty acids are used, and the formation of water-in-oil emulsions when the fatty acid salts of the alkaline earths or of the heavy metals are employed, is probably incorrect, inasmuch as the size of a molecule of a stabilizing soap is not sufficiently great to exert the leverage action

[27] Schlaepfer, A. U. M., Water-in-oil Emulsions, *J. Chem. Soc.*, 113 : 522–526 (1918).

[28] Hatschek, E., Emulsions, Second Report on Colloid Chemistry, Brit. Assoc. for the Advancement of Science (1918).

[29] Fischer, Martin H., and Hooker, Marian O., Fats and Fatty Degeneration, John Wiley & Sons, Inc., New York (1917).

[30] Donnan, F. G., and Potts, H. E., Ueber die Emulgierung von Kohlenwasserstoffölen durch wässerige Lösungen fettsaurer Salze, *Koll. Z.*, 7 : 208–214 (1910).

[31] Finkle, P., Draper, H. D., and Hildebrand, J. H., The Theory of Emulsification, Colloid Symposium Monograph, Vol. I, pp. 196–209 (Published by the Department of Chemistry, University of Wisconsin, Madison, 1923).

[32] Harkins, W. D., The Orientation of Molecules in the Surfaces of Liquids, Colliod Symposium Monograph, Vol. II, pp. 141–173, Chemical Catalog Company, Inc., New York (1925).

TABLE III

The Percentage Lowering of the Interfacial Tension between Pure Paraffin Oil and Water, Due to the Addition to the Aqueous Phase of Small Quantities of the Sodium Salts of a Homologous Series of the Saturated Aliphatic Acids.

Sodium Salt of	Number of Carbon Atoms	Lowering of Interfacial Tension	
		Concentration, $\frac{n}{400}$	Concentration, $\frac{n}{200}$
		Per Cent	Per Cent
Acetic acid	2	0.3	0.2
Propionic acid	3	0.6	2.3
Butyric acid	4	0.6	2.3
Valeric acid	5	0.5	2.0
Caproic acid	6	0.6	2.4
Oenanthylic acid	7	1.0	3.6
Caprylic acid	8	3.5	10.6
Pelargonic acid	9	4.1	13.8
Capric acid	10	9.2	20.4
Lauric acid	12	21.6	46.2
Myristic acid	14	41.0	

which is postulated, cf. Harkins.[33] On the other hand, there is no doubt but that in the case of emulsions stabilized by a soap, the hydrocarbon end of the molecule of the salt of a fatty acid is oriented toward the oil phase and the carbonyl group with its metallic radical is oriented toward the water phase. Figure 8 shows diagrammatically such orientation. In all probability the explanation of the change in form of the curves in Fig. 7 can be accounted for on the theories developed by Harkins,[34]

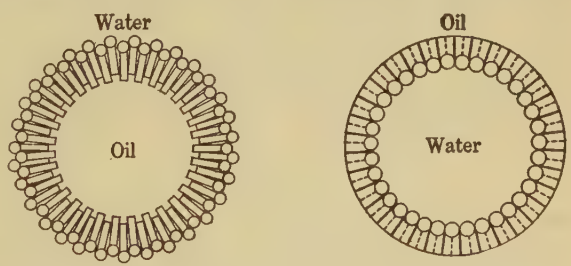


FIG. 8.—A diagrammatic representation of molecular orientation of the stabilizing molecules in oil-in-water and water-in-oil emulsions. (After Harkins.)

[33] Harkins, W. D., Colloid Symposium Monograph, Vol. II, pp. 141–173, Chemical Catalog Company, Inc., New York (1925).

[34] Harkins, W. D., Colloid Symposium Monograph, Vol. II, pp. 141–173 (1925); Surface Energy and Surface Tension, Chap. VIII of Colloid Chemistry, Theoretical

and Langmuir[35] dealing with the orientation of molecules in surface films.

This question of molecular orientation will be more fully considered in the following pages. It is sufficient to note at this point that the suggested explanation for the progressive reduction of surface tension in aqueous solutions of the alkali salts of the fatty acids with progressive increase in the length of the carbon chain is probably accounted for by the eventual formation of a hydrocarbon interface between liquid and air. When such an interface is complete and we have a continuous, closely packed " skin " of oriented molecules on the surface of the water, we no longer have a water-air interface but rather a hydrocarbon-air interface which, as will be noted by reference to Table III, has a much lower surface tension than a water-air interface.

As we increase the length of the carbon chain past C_{14}, there is relatively little change in the effect on surface tension or interfacial tension. This is to be expected, inasmuch as if we are already dealing with a hydrocarbon surface, the addition of a $-CH_2-$ radical underneath that surface should have but little effect on the properties of the surface. Clowes[36] has presented some very interesting experiments demonstrating the effect

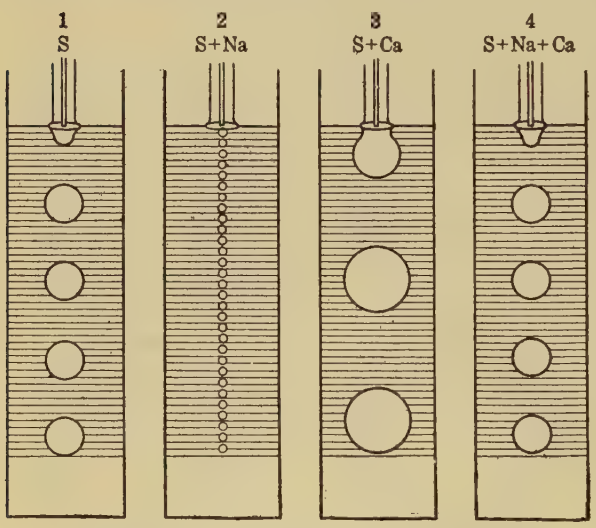


Fig. 9.—Illustrating Expts. 1–4 in Table IV. In each case the number of drops in the diagram is one-tenth of the number actually obtained. (After Clowes.)

and Applied, Vol. I, edited by Jerome Alexander, Chemical Catalog Company, Inc., New York (1926).

[35] Langmuir, Irving, The Effects of Molecular Dissymmetry on Some Properties of Matter, Chap. XXIX of Colloid Chemistry, Theoretical and Applied, Vol. I, edited by Jerome Alexander, Chemical Catalog Company, Inc., New York (1926); The Distribution and Orientation of Molecules, Colloid Symposium Monograph, Vol. III, pp. 48–75, Chemical Catalog Company, Inc., New York (1925).

[36] Clowes, G. H. A., Protoplasmic Equilibrium. I. Action of Antagonistic Electrolytes on Emulsions and Living Cells, *J. Phys. Chem.*, 20 : 407–451 (1916).

of sodium chloride and calcium chloride on emulsification. Figure 9 and Table IV, taken from the data of Clowes, represent the experimental results which were obtained. The experimental technic was to allow slightly rancid olive oil to flow from the tip of a stalag-

TABLE IV

Number of Drops Formed from a Given Volume of Olive Oil Dropping from a Standard Tip through Solutions of 0.001 M NaOH Alone and Plus Various Concentrations of NaCl and $CaCl_2$ Solutions.*

Exp. No.	Concentration of			Number of Drops	Interfacial Tension	
	NaOH	NaCl	$CaCl_2$		Olive Oil Dynes for Each Sq. Cm.†	Paraffin Oil. Dynes for Each Sq. Cm.†
1	0.001 M	.....	.....	44	7.3	7.2
2	0.001 M	0.15 M	.....	300	0.002	0.00
3	0.001 M	.....	0.0015 M	24	9.88	9.65
4	0.001 M	0.15 M	0.0015 M	44	6.88	7.48
5	0.001 M	0.30 M	0.003 M	43	6.36	7.12
6	0.001 M	0.45 M	0.005 M ‡	43	6.70	7.36
7	0.001 M	0.60 M	0.01 M §	43	7.31	8.20
8	.....	.....	.....	....	24.11	31.05

* Data of Clowes (1916) except for last two columns, which present the recent data of Harkins and Zollman (1926) on the interfacial tensions of these solutions.

† With 0.001 N oleic acid in the oil.

‡ 0.0045 in Harkins' experiments.

§ 0.0060 in Harkins' experiments.

mometer (an instrument for measuring surface tension if the liquid is allowed to drop through air, or interfacial tension if the tip is immersed under the surface of another liquid) and to count the number of droplets which are formed by a given volume of the oil. It will be noted that sodium chloride solutions greatly decrease interfacial tension with the resulting formation of small droplets, whereas calcium chloride solutions increase interfacial tension with the resulting formation of large droplets. The action of the sodium chloride and calcium chloride in the above experiments is undoubtedly explained by the formation of small amounts of sodium or calcium soaps, and while no emulsion resulted in the above case, it is obvious that the sodium salt increases the ease of dispersion of the olive oil in water, whereas the calcium salt would hinder dispersion.

It is of interest to note that sodium and calcium chlorides when mixed antagonize each other and nullify the effect of either, so that such solutions are without any great effect on interfacial tension. This phenomenon has become known in biological reactions as *antagonism of ions*. Thus, calcium antagonizes sodium, magnesium antagonizes calcium, and potassium antagonizes sodium. Whether or not all of these mutually neutralizing effects are due to interfacial tension changes has not been experimentally proven. However, Harkins and Zollman[37] have studied the systems investigated by Clowes in regard to interfacial tension changes, and the last two columns of Table IV show the results of their experimental data. The interfacial tension between olive oil and a dilute sodium chloride solution (Expt. 2, Table IV) is the lowest interfacial tension between oil and water which is recorded in the literature. It is obvious that even a slight agitation of such a system would result in the formation of a stable emulsion. Harkins and Zollman point out that when the interfacial tension of a water-benzene system falls below 10 dynes per square centimeter, the benzene can be easily emulsified in the aqueous phase and that when the interfacial tension falls below 1 dyne per square centimeter, emulsions are formed spontaneously. Such emulsions are stable for years.

The antagonistic action of calcium and sodium is well illustrated by the work of Chambers and Reznikoff.[38] Making use of Chambers' micromanipulation apparatus, amoebae were injected with dilute solutions of sodium chloride, potassium chloride, calcium chloride, and magnesium chloride. Injection of sodium chloride or potassium chloride caused a liquefaction of the protoplasm surrounding the injected area. In other words, the protoplasm shows a decreased viscosity, becoming more nearly a sol. On the other hand, when calcium chloride is injected, there is a local solidification of the injected protoplasmic region, producing a more or less rigid mass of gel which the amoeba " pinches off " and rejects. The portion affected is in this way eliminated, leaving the remainder of the amoeba in apparently a normal condition. When magnesium chloride is injected, there is a local solidification similar to that occurring on the injection of calcium chloride. In this case, however, no " pinching off " reaction takes place, and the solidification process gradually spreads throughout the amoeba, causing complete gelation and death.

[37] Harkins, W. D., and Zollman, Henrietta, Interfacial Tension and Emulsification. I. The Effects of Bases, Salts, and Acids upon the Interfacial Tension between Aqueous Sodium Oleate Solutions and Benzene. II. Extremely Small Interfacial Tensions Produced by Solutes, *J. Am. Chem. Soc.*, 48 : 69–80 (1926).

[38] Chambers, Robert, and Reznikoff, Paul, The Reaction of the Protoplasm of the Living Amoeba to Injected Salts, *Proc. Soc. Exp. Biol. and Med.*, 22 : 320–322 (1925).

When mixtures of sodium chloride and calcium chloride or when mixtures of potassium chloride and calcium chloride, similar in concentration to the mixtures used by Clowes and by Harkins and Zollman in Table IV, are injected, no effect is observable, the sodium chloride neutralizing the solidifying effect of the divalent salt and the divalent salt neutralizing the liquefying action of the sodium chloride. Chambers and Reznikoff suggest that " at least one of the features of the antagonistic action of NaCl or KCl to $CaCl_2$ is the maintenance in protoplasm of a definite balance between its liquid and solid phases. This phenomenon possibly depends upon the formation of a balanced proportion of Na and Ca or of K and Ca protein salts. It may also be due to the formation of Na or K and Ca soaps." In the light of the experiments of Harkins and Zollman we must conclude that interfacial tension phenomena and alteration in the type of protoplasmic emulsions are features in the experiments noted by Chambers and Reznikoff.

Weiser[39] has studied the antagonistic action of ions in the precipitation of colloid sols, with the interesting result that antagonistic actions similar to the observed biological phenomena have been demonstrated. He suggests that possibly the antagonistic action of salt pairs (*e.g.* Na *vs.* Ca) may be conditioned on the effect of the ions on the permeability of the colloidal film making up the cell membrane. The last word has not been written on the question of ion antagonism as exhibited in biological systems, but the solution of the phenomenon will undoubtedly be hastened when the methods of colloid chemistry are applied to such systems.

The breaking of emulsions and the inversion of emulsions involve changes in the interfacial tension between the two components and probably an alteration in the orientation of the surface film surrounding the suspended droplets. It sometimes happens that crude petroleum issues from the ground emulsified with the salt water associated with the petroleum fields. In such instances the emulsifying agent appears to be asphaltic residues. Obviously such emulsions are without commercial value, and the breaking of such emulsions becomes an important industrial problem. A knowledge of surface chemistry and of the role that interfacial tension plays in emulsion formation, as well as a knowledge of the antagonistic effects of ions, should prove very helpful to the chemist who is faced with such industrial problems.

As noted earlier, only a dilute emulsion results when we have oil dispersed in pure water. This is due to the fact that we have present in such a system a relatively high interfacial tension. Such systems behave as true lyophobic sols. When the interfacial tension is lowered by the

[39] Weiser, Harry B., The Antagonistic Actions of Ions in the Neutralization of Sols. II, *J. Phys. Chem.*, 30 : 1527–1537 (1926).

introduction of an emulsifying agent, an extremely high concentration of the disperse phase can be obtained. Such emulsions behave more or less as lyophilic systems and take on to a large degree the properties characteristic of the emulsifying agent. It is such systems that are usually called emulsions, and the fact that the stabilizing agent is generally a lyophilic colloid justifies our including emulsions in a consideration of colloid systems.

CHAPTER III

CERTAIN PHYSICAL PROPERTIES CHARACTERISTIC OF COLLOID SYSTEMS

Viscosity and Plasticity.—Viscosity may be defined as the internal friction of a liquid, the resistance to shear or flow. The unit of viscosity is the *poise*, so named from the Frenchman, Poiseuille,[1] who first devised methods for the measurement of viscosity. *A poise may be defined in cgs units as the force which, when exerted on a unit area between two parallel planes one square centimeter in area and one centimeter apart, produces a difference in streaming between the two planes of one centimeter velocity per second.* A centipoise, as the name implies, is $\frac{1}{100}$ of a poise, and inasmuch as the absolute viscosity of water at 20° C. is 1.0050 centipoise, the centipoise is generally used as the unit for plotting the viscosity of liquid systems. It may be noted from the above definition that viscosity is expressed in absolute terms and that it is not derived from a reference liquid. Too often there is a general misconception that water is the reference liquid to which viscosity units are referred. Bingham[2] has recorded the viscosity of various solutions which can be used as standard solutions for the calibration of an uncalibrated viscosimeter. Various types of viscosimeters are available.

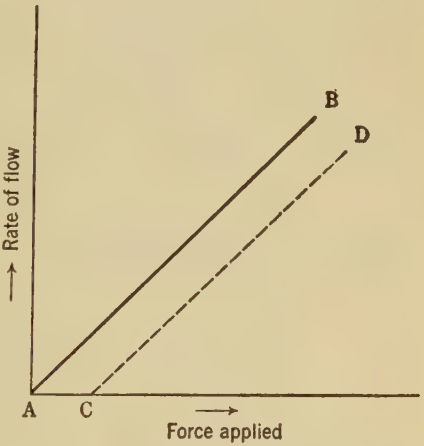


Fig. 10.—Showing diagrammatically the relation between true viscosity and plasticity. AC is the "yield value" of the plasticity formula.

The solid line (AB) in Fig. 10 is a diagrammatic representation of

[1] Poiseuille, Recherches expérimentales. Sur le mouvement des liquides de nature différente dans les tubes de très-petits diamètres. *Ann. chim. phys.*, 3 ser., 21 : 76–110 (1847).

[2] Bingham, E. C., and Jackson, R. F., Standard Substances for the Calibration of Viscometers, U. S. Bureau of Standards, Scientific Paper No. 298 (1917).

a truly viscous system. It will be noted that the increase in rate of flow with increase in force applied is a linear function which passes through the point of origin. The dotted line (*CD*) represents plastic flow, and it will be noted that it intersects the force axis to the right of zero force. It is accordingly necessary that energy be added to deform the system before plastic flow begins. This amount of energy is known as the *yield value*. Plasticity, therefore, differs from viscosity in that when we are dealing with plastic materials we must determine not only the rate of flow per unit of force applied, but likewise the yield value. This phase has been considered at length by Bingham and his coworkers.[3,4,5]

Colloid systems show a wide range in viscosity or plasticity. In general the lyophobic sols exhibit a viscosity which approaches very closely to the viscosity of the pure dispersions medium, and which increases only slightly with increasing concentration of dispersed material. On the other hand, lyophilic systems may reach very high values for viscosity, and many lyophilic sols and gels are truly plastic and have relatively high yield values. With lyophilic systems we do not have a linear relationship between viscosity or plasticity and the concentration of a disperse phase but rather a parabola, such as is shown diagrammatically in Fig. 11. This is undoubtedly due to the fact that the lyophilic systems are solvated, and accordingly the particle has associated with it a larger or smaller amount of the dispersions medium, so that the actual amount of the dispersions medium is decreasing with each added increment of disperse phase. Sharp and Gortner[6] have

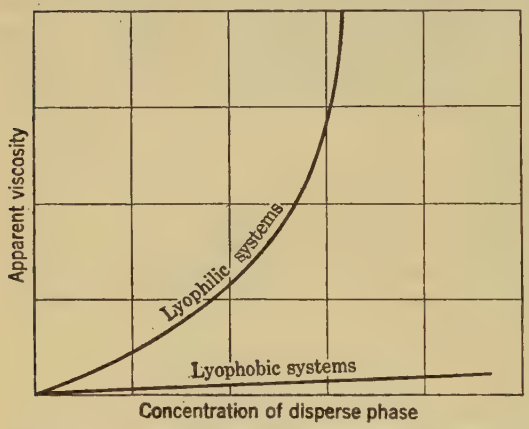


FIG. 11.—A diagrammatic representation of the relation between viscosity and concentration in lyophobic and lyophilic systems.

[3] Bingham, E. C., An Investigation of the Laws of Plastic Flow, U. S. Bur. of Standards, Sci. Paper No. 278 (1916).

[4] Bingham, E. C., Fluidity and Plasticity, McGraw-Hill Book Company, New York (1922).

[5] Bingham, E. C., and Durham, T. C., The Viscosity and Fluidity of Suspensions of Finely-Divided Solids in Liquids, *Am. Chem. J.*, 46 : 278-297 (1911).

[6] Sharp P. F., and Gortner, R. A., Viscosity as a Measure of Hydration

summarized certain of the literature dealing with the viscosity of colloidal systems. Excellent discussions have also been presented by Ostwald,[7,8] and by Hatschek.[9–13] Bingham[14] has likewise given a general review.

As a general rule the viscosity of colloid systems decreases as temperature increases. This is due in part to the effect of temperature on the dispersions medium. Water, for example, has a viscosity of 0.2838 centipoise at 100° C., whereas at 0° C. it has a viscosity of 1.7921 centipoise. It will be noted that the viscosity has increased approximately 800 per cent from 100° to 0°. Accordingly one reason why a precipitate can be washed more readily with boiling water than with cold water lies in the fact that hot water passes more readily through the pores of the filter paper, due to the lowered viscosity of the water. The question as to whether or not a precipitate should be washed with hot water or with cold water depends upon two factors, the change in solubility of the material with temperature and the change of viscosity of water with temperature. If the change in the viscosity of the water is greater than the change in solubility, more efficient and more rapid washing can be effected with a given volume of water by using hot water instead of cold water. On the other hand, if the change in temperature increases the solubility of the precipitate more rapidly than the corresponding viscosity changes, the hot water should be avoided in order to obtain the maximum yield of the desired precipitate.

With colloid systems, viscosity changes due to temperature are influenced not only by the viscosity of the dispersions medium but like-

Capacity of Wheat Flour and its Relation to Baking Strength, Minnesota Agricultural Experiment Station Technical Bulletin No. 19 (1922).

[7] Ostwald, Wo., The Importance of Viscosity for the Study of the Colloidal State, *Trans. Faraday Soc.* 9 : 34–46 (1913).

[8] Ostwald, Wo., Ueber die Bedeutung der Viskosität für das Studium des kolloiden Zustandes, *Koll. Z.*, 12 : 213–222 (1913).

[9] Hatschek, E., Die Viskosität der Dispersoide, *Koll. Z.*, 7 : 301–304 (1910).

[10] Hatschek, E., Die allgemeine Theorie der Viskosität zweiphasiger Systeme, *Koll. Z.*, 12 : 238–246 (1913).

[11] Hatschek, E., The General Theory of Viscosity of Two Phase Systems, *Trans. Faraday Soc.*, 9 : 80–92 (1913).

[12] Hatschek, E., The Viscosity and Hydration of Colloidal Solutions, *Biochem. J.*, 10 : 325–330 (1916).

[13] Hatschek, E., The Viscosity of Colloidal Solutions, Chap. XLVI of Colloid Chemistry, Theoretical and Applied, Vol. I, edited by Jerome Alexander, Chemical Catalog Company, Inc., New York (1926).

[14] Bingham, E. C., Fluidity and Plasticity, Chap. XLIV of Colloid Chemistry, Theoretical and Applied, Vol. I, edited by Jerome Alexander, Chemical Catalog Company, Inc., New York (1926).

wise by the effect of temperature on solvation. Most lyophilic colloids are more highly solvated at the lower temperatures. Thus, for example, gelatin and agar form relatively non-viscous sols at the higher temperatures but set to more or less rigid or plastic gels at the lower temperatures. Starch, on the other hand, forms a more or less lyophobic suspension at the lower temperatures, and the temperature may be increased appreciably without great changes in viscosity until a critical temperature, known as the gelatinization point, is reached. At this critical point the starch granules undergo rapid hydration and the extremely viscous or plastic starch paste results. An increase in temperature past this point results in a decreased viscosity or plasticity.

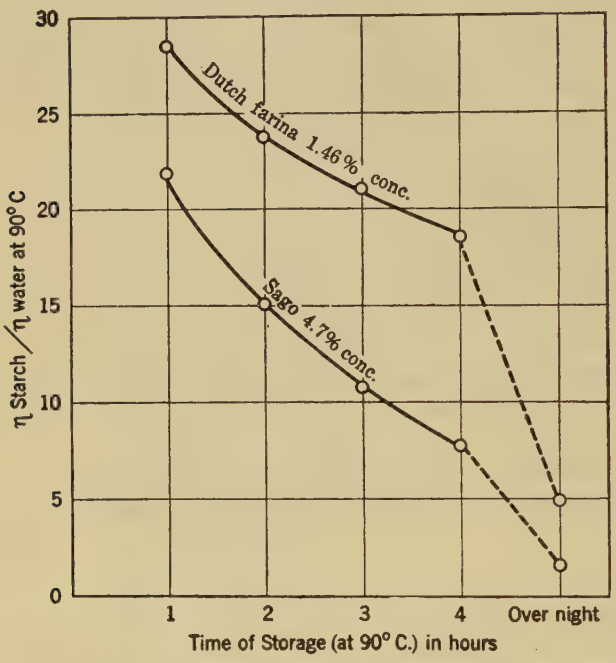


FIG. 12.—Showing changes in relative viscosity of gelatinized starch systems with time. (Data of Farrow and Lowe.)

Time may have a great effect on plasticity or viscosity of colloid systems. Farrow and Lowe[15] made an investigation of the changes in viscosity of a starch paste with time. Figure 12 shows a typical curve taken from their data. This change in viscosity is undoubtedly associated with a decrease in the solvation of the micelles.

Electrolytes may greatly alter the viscosity of lyophilic systems, in some instances causing relatively enormous changes. In order for such results to be manifest, it is essential that colloid systems be as nearly as possible electrolyte-free. Figure 13, taken from the data of Sharp and Gortner[16] shows the enormous decrease in the viscosity of an acidulated

[15] Farrow, F. D., and Lowe, G. M., The Flow of Starch Paste through Capillary Tubes, *J. Textile Institute*, 14 : 414–440 (1923).

[16] Sharp, P. F., and Gortner, R. A., The Physico-chemical Properties of Strong

wheat flour-water system on the addition of a trace of the salt of a bivalent metal. Similar effects are common when biochemical systems are under investigation.

The viscosity of a crystalloidal solution of a given solute in a given solvent is determined solely by the concentration and the temperature at which the measurement is made. This is not true for lyophilic colloid systems. Ostwald[17, 18] has pointed out that in lyophilic systems there are at least ten factors which must be taken into consideration, (1) concentration, (2) temperature, (3) degree of dispersion, (4) solvation, (5) electrical charge, (6) previous thermal treatment, (7) previous mechanical treatment, (8) the presence or absence of traces of other lyophilic colloids, (9) the age of the lyophilic sol, and (10) the presence of both electrolytes and non-electrolytes. As Sharp and Gortner have pointed out, another factor of extreme importance should be added to this list, *i.e.*, the rate of shear. It is obvious from the above list of variables that the study of viscosity or plasticity in lyophilic systems presents experimental difficulties. On the other hand, viscosity and plasticity methods afford one of the most valuable tools available to the colloid chemist.

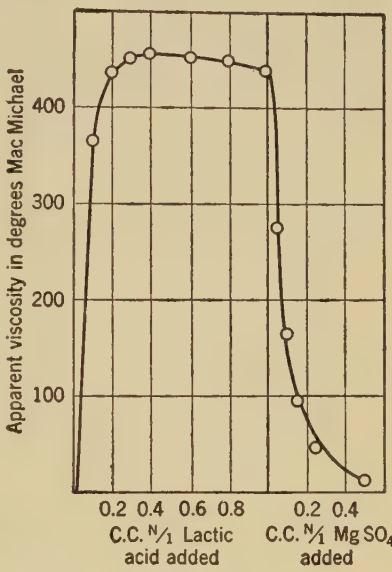


FIG. 13.—Showing the changes in apparent viscosity produced in a flour-water suspension by acidulation followed by the addition of an inorganic salt solution. (Data of Sharp and Gortner.)

In contrast to the linear relationships of truly viscous flow, the colloid chemist is continually discovering unexpected changes in the apparent viscosity of the systems with which he deals. Figure 14, taken from the data of Wo. Ostwald,[19] illustrates the peculiar form of the viscosity

and Weak Flours. V. The Identity of the Gluten Protein Responsible for the Changes in Hydration Capacity Produced by Acids, *J. Phys. Chem.*, 27 : 674–684 (1923).

[17] Ostwald, Wo., The Importance of Viscosity for the Study of the Colloidal State, *Trans. Faraday Soc.*, 9 : 34–46 (1913).

[18] Ostwald, Wo., Ueber die Bedeutung der Viskosität für das Studium des kolloiden Zustandes, *Koll. Z.*, 12 : 213–222 (1913).

[19] Ostwald, Wo., *Koll. Z.*, 12 : 217 (1913).

curve yielded by egg white during the process of heat coagulation. The reasons for the very peculiar curve are still unexplained. Figure 15, likewise taken from the same paper by Wo. Ostwald, shows the viscosity curve for the gelatinization of starch. No technic other than viscosity or plasticity measurements is delicate enough to indicate the enormous changes which take place in the colloidal properties of systems such as those illustrated in these figures.

Poiseuille first developed from theoretical considerations a formula

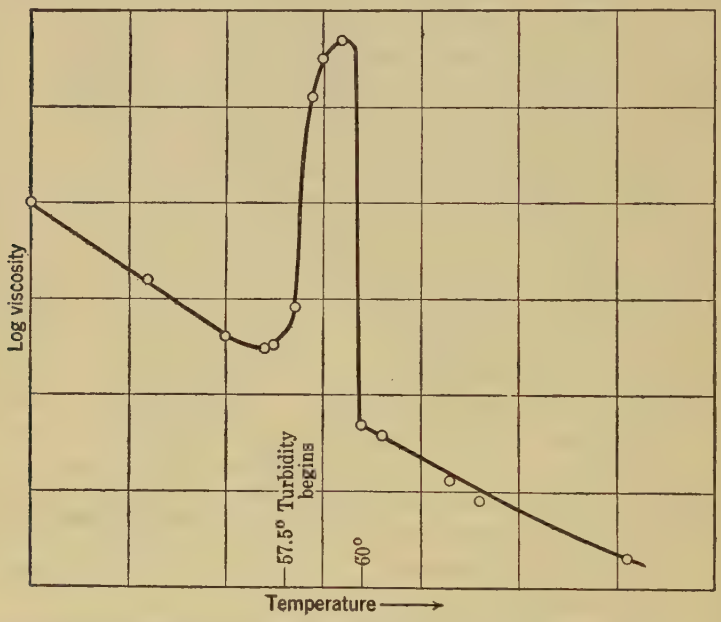


FIG. 14.—Showing viscosity changes which accompany the heat-coagulation of egg albumin. (Data of Wo. Ostwald.)

for viscosity based on the flow of a liquid through a capillary tube, on the theory that such a column of liquid was composed of innumerable cylinders, each moving slightly faster than the other as the distance from the wall of the tube increased. Stokes,[20, 21] and Hagenbach[22] modified

[20] Stokes, G. G., On Some Cases of Fluid Motion, *Trans. Camb. Phil. Soc.*, 8 : Pt. I 105–137 (1844).

[21] Stokes, G. G., On the Theories of the Internal Friction of Fluids in Motion and of the Equilibrium and Motion of Elastic Solids, *Trans. Camb. Phil. Soc.*, 8 : Pt. III, 287–319 (1847).

[22] Hagenbach, E., Ueber die Bestimmung der Zähigkeit einer Flüssigkeit durch den Ausfluss aus Röhren, *Pogg. Ann. Phys. Chem.*, 109 : 385–426 (1860).

the original formula of Poiseuille to the formula now generally accepted as representing truly viscous flow:

$$\eta = \frac{\pi r^4 pt}{8lV} \tag{4}$$

where η = the coefficient of viscosity;
r = the radius of the tube;
p = hydrostatic pressure of the liquid flowing through the tube;
t = the time necessary for the volume (V) to flow through the capillary;
l = length of the capillary.

In unit time (one second) this formula becomes:

$$V = \frac{\pi r^4 p}{8l\eta} \tag{5}$$

It will be noted that the time necessary for the liquid to flow through the capillary is inversely proportional to the hydrostatic pressure. This formula is very satisfactory for pure liquids and for truly crystalloidal solutions. Even in these cases, however, deviations from the formula may be observed at high pressures, in which case the flow is more like a solid rod of liquid being forced through without the accompanying internal friction, a film of liquid practically without motion acting as a lubricant on the walls of the tube.

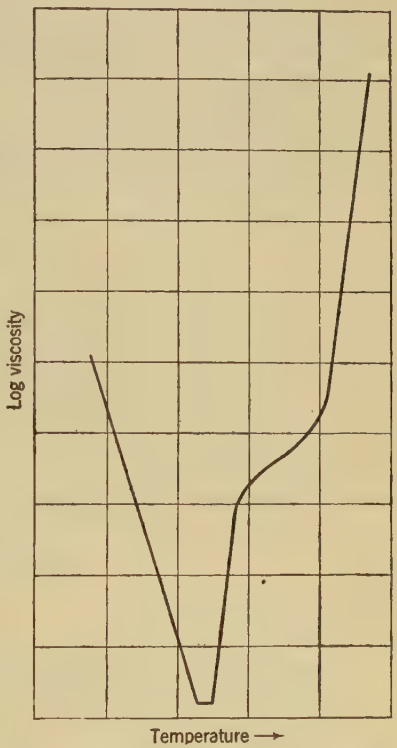


Fig. 15. — Showing viscosity changes which accompany the heat-gelatinization of starch. (Data of Wo. Ostwald.)

The material comprising the walls of the capillary tube makes no appreciable difference in the viscosity of truly viscous systems. Apparently there is always a thin film of liquid on the surface of the capillary tube, so that friction between the liquid and the wall of the capillary does not exist. The friction is actually between the bulk of the liquid flowing through the tube and a very thin film of liquid fixed on the surface of the capillary.

Recently torsion viscosimeters[23, 24] have been perfected which show particular adaptibility to rapid determinations of the viscosity of industrial materials. The principle of the torsion viscosimeter is to rotate the liquid in question in a cup at a constant rate of speed, at the same time suspending a cylinder in the liquid by means of a torsion wire. The friction of the rotating liquid on the cylinder immersed in it twists the torsion wire a certain number of degrees. The torc of the wire measured in angular degrees can be related to the viscosity of the liquid.

No adequate formula has been devised which expresses the viscosity of a lyophilic system. Hatschek proposed the formula,

$$\eta' = \eta \frac{\sqrt[3]{\phi}}{\sqrt[3]{\phi - 1}} \tag{6}$$

where η' = viscosity of the lyophilic sol;
η = viscosity of the dispersions medium;
ϕ = ratio of space occupied by the total volume of the system to the volume occupied by the disperse phase.

Hatschek[25-27] states that this formula is only a first approximation. Another formula[28, 29] which has been suggested is

$$\eta' = \eta(1 + K\phi) \tag{7}$$

where K = a constant.

Hatschek estimated that the constant had a value of 4.5. Einstein at first suggested that the constant had a value of 1.0 but on later calculations gave it a value of 2.5. Here again we are dealing with a formula which at its best is only a first approximation. Kunitz[30]

[23] Hatschek, E., Die allgemeine Theorie der Viskosität zweiphasiger Systeme, *Koll. Z.*, 12 : 238–246 (1913).

[24] Herschel, W. H., The Macmichael Torsional Viscosimeter, *J. Ind. Eng. Chem.*, 12 : 282–286 (1920).

[25] Hatschek, E., Die Viskosität der Dispersoide, *Koll. Z.*, 7 : 301–304 (1910).

[26] Hatschek, E., Die allgemeine Theorie der Viskosität zweiphasiger Systeme, *Koll. Z.*, 12 : 238–246 (1913).

[27] Hatschek, E., Die Viscosität von Blutkörperchen-Suspensionen. *Koll. Z.*, 27 : 163–165 (1920).

[28] Einstein, A., Ein neue Bestimmung der Moleküldimensionen. *Ann. Physik.*, 4 Folge, 19 : 289–306 (1906).

[29] Einstein, A., Berichtigung zu meiner Arbeit "Eine neue Bestimmung der Moleküldimensionen," *Ann. Physik.*, 4 Folge, 34 : 591–592 (1911).

[30] Kunitz, M., An Empirical Formula for the Relation between Viscosity of Solution and Volume of Solute, *J. Gen. Physiol.*, 9 : 715–725 (1926).

has suggested that the formula is probably more accurate when expressed as,

$$\eta = \frac{1 + 0.5\phi}{(1 - \phi)^4} \tag{8}$$

and finds that this formula expresses fairly accurately the relation between the volume of the solute and the viscosity of the system.

When we pass to plastic systems we must take into consideration the yield value. As already noted, the yield value indicates the pressure which must be exerted before the theoretical flow will start. The term, *consistency*, or its reciprocal, *mobility*, is used in place of viscosity to indicate that we are dealing with plastic flow. In its simplest terms the formula for viscosity may be expressed as,

$$\frac{V}{P} = K \tag{9}$$

where V = volume of liquid passing through the capillary per second;
P = pressure in grams per square centimeter producing the flow;
K = the constant.

This formula in terms of plasticity becomes

$$\frac{V}{P - p} = K_1 \tag{10}$$

where p = yield value pressure in grams per square centimeter.

Bingham and White[31] give a somewhat more elaborate formula for viscous flow.

$$\eta = \frac{\pi G r^4 P t}{8V(l + \lambda)} - \frac{MDV}{8\pi t(l + \lambda)} \tag{11}$$

where η = coefficient of viscosity;
G = gravitational constant;
P = pressure in grams per square centimeter;
t = time in seconds;
V = volume of flow;
r = radius of capillary in centimeters;
l = length of capillary in centimeters;
D = density of the liquid;

[31] Bingham, E. C., and White, G. F., Fluidität und die Hydrattheorie. I. Die Viskosität von Wasser, *Z. physik. Chem.*, 80 : 670–686 (1912).

M = a constant whose value was taken as 1.12 by Bingham and White;

λ = a correction to be added to the length of the capillary to correct for the viscous flow outside of the capillary.

The expression, $\frac{MDV}{8\pi t(l+\lambda)}$, is subtracted to correct for the kinetic energy of the liquid emerging from the capillary. This correction is usually extremely small, approaching zero, and may be disregarded in a consideration of plastic flow. Neither is it necessary to correct for the resistance to flow outside the capillary, so that the equation for plastic flow may be written,

$$\text{Consistency} = \frac{\pi r^4 G(P-p)t}{8lV} \tag{12}$$

Plasticity is usually expressed in terms of mobility (μ) which is the reciprocal of the consistency. Kelly[32] has derived a somewhat different formula which he has applied to the study of plasticity of rubber sols, and suggests that by altering the method of measurement and using a differential equation, fluidity values accurate to within 1 per cent can be obtained. Kelly's original paper should be consulted for the details of the method. His equation for fluidity is

$$\phi_2 = \frac{\frac{\Delta V}{\Delta t}}{\frac{\pi P r^4}{8\frac{(l_2+l_1)}{2}}} \tag{13}$$

where ϕ_2 = the fluidity at a point midway between two calibrations, l_1 and l_2, on the capillary tube, the other terms in the equation being identical with the terms used in the equation above.

Sharp[33] has applied plasticity methods to a study of flour-in-water suspensions similar to those studied earlier by Sharp and Gortner, and finds that even simple flour-in-water suspensions are plastic when they contain 9 per cent or more of flour by weight on the dry basis. The introduction to Sharp's paper summarizes some of the most important literature dealing with plastic flow.

[32] Kelly, W. J., The Plasticity of Rubber and its Sols. I. Colloid Symposium Monograph, Vol. III, pp. 303–316 (1925).

[33] Sharp, P. F., Wheat and Flour Studies V. Plasticity of Simple Flour-in-water Suspensions, *Cereal Chem.*, 3 : 40–56 (1926).

Hysteresis.—Hysteresis, in colloid terminology, is not synonymous with the same term used in engineering practice. Hysteresis is used by the colloid chemist in expressing in a single term the influence of the previous history of a colloid system on its present behavior. It has been allegorically referred to as the " memory " of a colloid system. In many instances the past history of a colloid system very largely determines its present behavior. This is especially true when we are dealing with lyophilic gels. Bancroft[34] noted some unpublished preliminary results of Cartledge, showing the effect of past history on the behavior of dried gelatin films. This citation caused Gortner and Hoffman[35] to make a study of the effect of the concentration of an original gelatin gel on the behavior of dried gelatin prepared from such gels. Weighed quantities (10, 15, 20, 25, 35, and 40 grams) of " Bacto " gelatin [36] were added to 100 cc. of distilled water in clean pyrex flasks. After soaking for fifteen to thirty minutes the flasks were placed in a hot water bath and allowed to remain until all of the gelatin had dissolved to form a homogeneous solution. A measured quantity (25 cc.) of this solution was then poured into petri dishes 89 mm. in diameter, thus ensuring the same thickness of the gelatin gel in each instance. After standing for twelve hours, duplicate rectangles 5×2.5 cm. in surface area were cut and placed on watch glasses to dry in a current of warm air (30°–40° C.) to a moisture content which did not exceed 3.5 per cent. The rate of moisture loss was followed by frequent weighings of the gelatin plates during the drying process, but no marked differences in rate of moisture loss were observed. The dried sections were then placed in distilled water and allowed to reimbibe moisture. The rate of swelling was followed by weighing the discs at frequent intervals after removing surface water by blotting with neutral filter paper. The data calculated in *grams water imbibed per gram dry gelatin* for the 10, 25, and 40 per cent gels are shown in Fig. 16. In order to be perfectly sure that the shape or thickness of the gelatin plate was not responsible for the differences in swelling, portions of dried plates from 10-gram, 25-gram, and 35-gram gels, as well as portions of the " original " Bacto gelatin, were ground and sieved, those particles passing through a 2-mm. sieve and remaining on a 1-mm. sieve being retained for experimental work. A weighed quantity of these " granules " was placed in a Gooch crucible and allowed to imbibe water. At frequent intervals

[34] Bancroft, W. D., Applied Colloid Chemistry, p. 251, McGraw–Hill Book Company, New York (1921).

[35] Gortner, R. A., and Hoffman, W. F., Evidence of a Structure in Gelatin Gels, *Proc. Soc. Exp. Biol. and Med.*, 19 : 257–264 (1922).

[36] Air-dry gelatin as received from manufacturer.

the crucibles were removed from the water, centrifuged at low speed for two minutes in order to remove excess moisture, and weighed. Figure 17 shows the form of the imbibition curves.

Later Gortner and Hoffman[37] studied the hysteresis effects in dried gelatin, part of which had been dried from gelatin sols at 45° C., the remainder having been dried at a temperature below the gelating point, so that it had remained in the gel form throughout the entire period of drying. Table V shows the results which were obtained. It is obvious

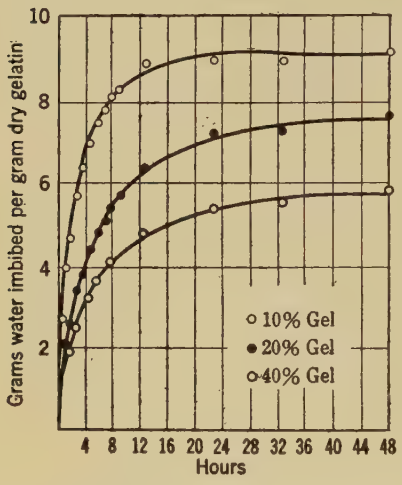


Fig. 16.—Showing imbibition curves of gelatin which had been dried down to approximately 3 per cent moisture content from gels of different initial concentrations and then again allowed to swell in water. (Data of Gortner and Hoffman.)

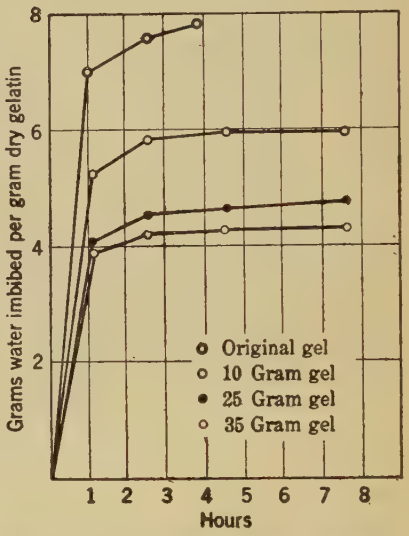


Fig. 17.—Imbibition curves of gelatin, similar to those shown in Fig. 16 except that these curves are for granules of uniform size.

from these experiments that the previous treatment has a profound effect on the rate of hydration of gelatin particles. Similarly, if gelatin gels are " melted " and allowed to resolidify, and this process is repeated a number of times, it will be found that the " melting point " temperature becomes progressively lower and the " solidifying point " temperature is likewise lowered. In a similar manner, rubber can be stretched and released a number of times so that it eventually changes its shape and becomes permanently distorted. Starch pastes on standing undergo retrogression with a loss in viscosity or plasticity and a great loss in water-imbibing capacity (cf. Fig. 12).

[37] Gortner, R. A., and Hoffman, W. F., The Imbibition of Gelatin Dried as a Gel and as a Sol, *J. Phys. Chem.*, 31 : 464–466 (1927).

TABLE V

THE IMBIBITION OF UNIFORM SIZED PARTICLES OF GELATIN DRIED AS A "GEL" AND AS A "SOL"

Source of Gelatin Particles, Per Cent	Grams Water Imbibed per Gram Dry Gelatin *							
	1 Hr.	2 Hrs.	3 Hrs.	4 Hrs.	6 Hrs.	8 Hrs.	12 Hrs.	24 Hrs.
Gelatin Sol.								
5	9.12	10.98	11.97	12.60	12.94	13.69	15.32	16.50
10	3.69	5.65	6.63	7.35	8.23	9.30	10.78	12.13
15	3.64	5.02	5.72	6.45	7.54	8.95	10.76	12.18
20	3.64	4.89	5.73	6.44	7.60	8.70	10.10	11.32
Gelatin Gel.								
5	7.27	9.72	10.97	11.75	12.81	14.16	17.17	18.46
10	5.33	7.03	8.21	9.00	10.28	11.38	13.71	14.63
15	5.32	6.70	7.59	8.48	9.12	10.52	12.10	13.35
20	4.19	5.88	6.84	7.45	8.50	9.88	12.06	12.96

*Average of duplicate determinations. In this experiment the gelatin was ground to pass a 2 mm. mesh and was all retained on a 1 mm. mesh.

The phenomenon of hysteresis undoubtedly plays a very great role in biological organisms, and when the individual factors are ultimately analyzed, it will certainly be found that the behavior of the lyophilic colloids which are present is predominantly responsible for the reactions which are observed. Hysteresis effects are most striking when one uses viscosity or plasticity methods. Accordingly, in using such methods, the past history of the colloidal system under investigation may well be the most important variable, and it is an essential precaution to duplicate, insofar as possible, every detail of mechanical, thermal, or electrical treatment, as well as to observe exact time intervals, if one is to obtain reliable data regarding the viscosity or plasticity of lyophilic colloid systems. Apparently the most striking effects of hysteresis are noted in studies of degree of solvation.

BROWNIAN MOVEMENT.—In 1827, a botanist, Robert Brown noted that pollen grains suspended in a liquid on the microscope stage were in continuous motion in the liquid. In order to ascertain whether or not such motion was characteristic of living pollen grains, he secured from the herbarium, pollen and plant spores which were more than one hundred years old and found that the rate of motion was in no way diminished. It was later observed that any particle of small enough size to remain in more or less permanent suspension in a liquid would exhibit this characteristic motion which has been called the Brownian

movement. Particles up to 4μ and accordingly easily visible in the microscope show the characteristic Brownian motion, but the motion is not as active or as rapid as it is in colloidal systems.

Burton[38] has given an excellent presentation of the historical phases and theoretical significance of Brownian movement.

A particle in a Brownian movement oscillates rapidly in a haphazard manner around a mean position so that the velocity of movement must be calculated from observations occupying very short periods of time. This has been done by Svedberg, who employed a motion picture camera to record changes in the position of a particle. Measurements of displacement on the film indicate that particles 40–50$m\mu$ in diameter in a platinum hydrosol may reach a velocity as great as 320μ per second. This velocity in relation to the size of the platinum particle approximates the velocity of a low-speed rifle ball.

Various theories were devised in order to account for Brownian movement. These theories have been adequately discussed by Burton. It is now definitely known that Brownian movement is caused by the kinetic energy of the liquid in which the particle is suspended. Gouy[39,40] states that "the Brownian movement of all the physical phenomena shows us visibly that there is a constant state of internal agitation in liquids even in the absence of all external causes." Many striking experiments have been performed to test the kinetic theory of Brownian motion. Perhaps the most striking experiments are those of Perrin[41-44] who pointed out that particles of identical size and shape suspended in a liquid distributed themselves according to the same law as is observed by molecules of a gas acted upon by gravity, *i.e.*, the particles are less dense at the surface of a liquid and become more dense as the depth below the surface increases. Perrin counted in a single experiment as many as 13,000 particles at varying depths of 5μ, 35μ, 65μ, and 95μ below the surface and found that the number of particles increased approximately in geometrical progression. As Burton notes, the concentration drops to about one half value in a change of 0.03 mm. difference in level, whereas

[38] Burton, E. F., The Physical Properties of Colloidal Solutions, Second Edition, Chapter IV, Longmans, Green and Company, London (1921).

[39] Gouy, M., Note sur le mouvement Brownien, *J. de Phys.*, 7 : 561–564 (1888).

[40] Gouy, M., Sur le mouvement Brownien, *Compt. rend.*, 109 : 102–105 (1889).

[41] Perrin, J., L'origine du mouvement Brownien, *Compt. rend.*, 147 : 530–546 (1908).

[42] Perrin, J., Grandeur des molécules et charge de l'electron, *Compt. rend.*, 147 : 594–596 (1908).

[43] Perrin, J., Mouvement Brownien et réalité moléculaire, *Ann. chim. phys.*, [8] 18 : 5–114 (1909).

[44] Perrin, J., Wie wägt man ein Atom? *Z. Elektrochem.*, 15 : 269–277 (1909).

in the atmosphere a similar decrease would require a difference in level of 6,000 kilometers.

Figure 18 represents the distribution of particles of gamboge and mastic at different levels in their respective sols.

Burton and Currie[45] have recently pointed out that Perrin's distribution is limited to a very thin surface layer less than 0.1 mm. in depth. Below this layer the distribution of the particles in the sol is uniform.

Perrin, finding that the distribution ratios of the various particles at different depths in the surface layer obeyed the gas laws, determined Avogadro's constant for his colloidal sols, and from the formula

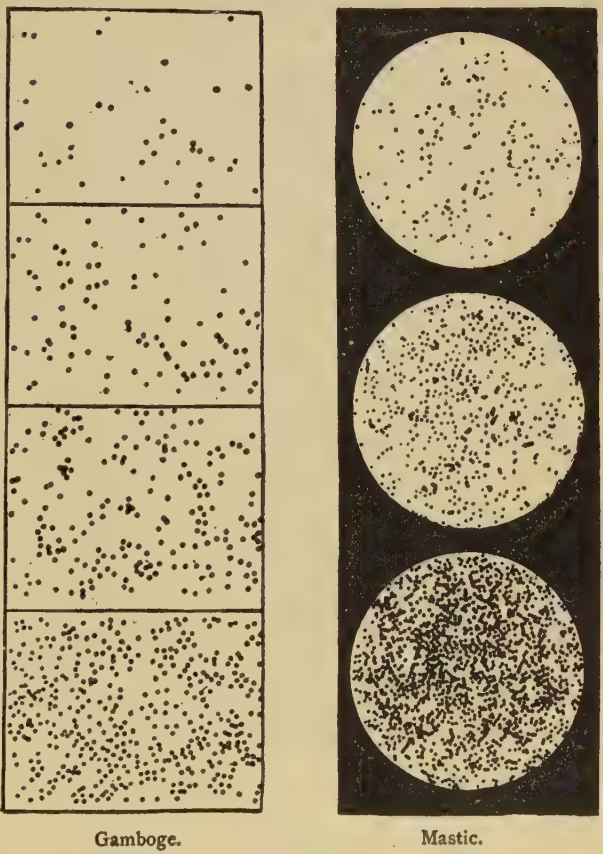


FIG. 18.—A diagrammatic representation of Perrin's experiment on the distribution of particles in a sol. (After Burton.)

$$N = \frac{3}{2}\left(\frac{RT}{W}\right) \tag{14}$$

where N = the number of particles (molecules in the case of a gas) in 1 gm. molecule;

W = the mean kinetic energy of a particle (the mean kinetic energy of a molecule in a gas),

he found a value of $N = 70.5 \times 10^{22}$.

[45] Burton, E. F., and Currie, J. E., The Distribution of Colloidal Particles, *Phil. Mag.*, 47 : 721–724 (1924).

Smoluchowski devised a formula based on the displacement of a particle under Brownian movement, due to the kinetic energy of the disperse phase, and Einstein independently developed a similar formula for the motion of small spheres suspended in a liquid medium. Their formulae were based on the assumption that the individual particle behaved as a gas molecule and that the gas laws could accordingly be applied. Their formulae yielded values for N of 70.5×10^{22} and 65.0×10^{22}, respectively. These values are strikingly in agreement with the values of Millikan, and Rutherford, calculated from the direct determination of the electrical charge on ions, *i.e.*, 61.8×10^{22} and 62.3×10^{22}, respectively. We must accordingly note that colloidal systems obey the gas laws of physical chemistry, providing that each particle of the disperse phase is regarded as behaving as a single molecule. A gram molecular weight of Perrin's gamboge sol containing 60.61×10^{22} particles would have a " molecular " weight of 30,000,000,000 or approximately 350,000 tons, and this amount of disperse phase would have to be contained in one liter of dispersions medium in order to show a normal osmotic pressure and other properties characteristic of a normal solution. Obviously there is no such thing as a molecular weight of 30,000,000,000. The " molecular weight " of colloid micelles is a misnomer, the more appropriate term being particle weight. In biochemical literature molecular weights of proteins as high as 200,000 are recorded. Probably here the more correct term would be particle weight.

Brownian movement undoubtedly tends to stabilize a colloid system. According to Stokes' Law of falling bodies,

$$V = \frac{2}{9}\left(\frac{D-d}{\eta}\right) Gr^2 \qquad (15)$$

where V = the velocity of fall;
D = the specific gravity of the falling particle;
d = the specific gravity of the medium through which the fall takes place;
η = the viscosity of the medium;
G = the gravity constant;
r = the radius of the particle.

every substance differing in specific gravity from the medium in which it is suspended will be drawn through that medium by the force of gravity. Applying the above formula to a gold particle $10m\mu$ in diameter, we find a velocity of fall of approximately 1 centimeter per month. Inasmuch as Brownian movement causes a very much greater displacement of the particle than does the force of gravity, Brownian movement must be

regarded as a stabilizing agent, although if all convection currents were eliminated and all mechanical vibration controlled, there would be a very slow but inevitable settling of the colloidal particles.

Burton[46] has made calculations of the velocity of Brownian movement and the rate of settling, according to Stokes' law, of silver particles having different radii. His calculations are shown in Table VI. It will be noted that for particles having a diameter greater than 100×10^{-7} cm. ($100m\mu$) the velocity of sedimentation exceeds the velocity of Brownian movement. However, at $50m\mu$ the velocity of Brownian movement is approximately three times the velocity of sedimentation, and at the lower limit of the colloid realm it is 60,000 times the velocity of sedimentation.

TABLE VI

Showing the Relative Velocity Due to (*a*) Brownian Movement and (*b*) Settling According to Stokes' Law under the Pull of Gravity, of Silver Particles Suspended in Air (Calculations of Burton).

Radius of Particle	Velocity of Brownian Movement	Velocity of Sedimentation
Cm. $\times 10^{-7}$	Cm. $\times 10^{-7}$ per Second	Cm. $\times 10^{-7}$ per Second
1000	2,000	1,200,000
500	2,800	300,000
100	6,300	12,000
50	8,900	3,000
10	20,000	120
5	28,000	30
1	63,000	1.2

Inasmuch as the calculations in Table VI are for silver particles suspended in air, the silver having a specific gravity of 10.5 and air a viscosity of only 1.9×10^{-4}, the sedimentation velocity is very much greater than it would be for a liquid system or for particles of organic materials where the specific gravity approaches the specific gravity of the dispersions medium. If we were dealing with organic particles dispersed in water, the velocity of Brownian movement would exceed the velocity of sedimentation when the radius of the particle was considerably greater than $100m\mu$.

Svedberg and coworkers[47-50] have applied Stokes' law to the deter-

[46] Burton, E. F., The Physical Properties of Colloidal Solutions, Second Edition, Longmans, Green and Co., New York (1921).

[47] Svedberg, The, and Rinde, H., The Ultracentrifuge, a New Instrument for the

mination of the size of colloidal micelles by devising an ultracentrifuge which permits them to study the rate of movement of colloidal particles under a greatly increased gravitational force. Knowing the gravitational force which is applied, they are able to calculate the radius of the particle and accordingly the particle weight of the suspended material.

Wa. Ostwald[51] has devised a nomograph chart showing the relation of particle size to rate of settling and accordingly to colloidal stability. Anyone knowing the diameter of the suspended particles and their specific gravity can readily determine by the use of this chart how long the suspension will be stable, or inversely, knowing the rate of settling and the specific gravity, the diameter of the particles in question can be ascertained. That studies of Brownian movement have more than theoretical interest, is illustrated by the papers of Tolman and others,[52-56] who applied the theoretical methods of earlier workers in this field to a study of the efficiency of materials for the production of obscuring smokes to be used in military operations. Figure 19 is a reproduction of a photomicrograph illustrating the paper of Wells and Gerke, taken under conditions where a particle in Brownian movement was allowed to flow slowly across the field.

Osmotic Pressure of Colloid Systems.—As already noted, the gas laws can be applied to colloidal systems, providing each particle

Determination of Size and Distribution of Size of Particle in Amicroscopic Colloids, *J. Am. Chem. Soc.*, 46 : 2677–2693 (1924).

[48] Svedberg, The, Zentrifugierung, Diffusion und Sedimentationsgleichgewicht von Kolloiden und hochmolekularen Stoffen, *Koll. Z., Zsigmondy Festschrift*, 53–64 (1925).

[49] Svedberg, The, and Fahraeus, R., A New Method for the Determination of the Molecular Weight of the Proteins, *J. Am. Chem. Soc.*, 48 : 430–438 (1926).

[50] Svedberg, The, and Nichols, J. B., The Application of the Oil Turbine Type of Ultracentrifuge to the Study of the Stability Region of Carbon Monoxide-Hemoglobin, *J. Am. Chem. Soc.*, 49 : 2920–2934 (1927).

[51] Ostwald, Wa., Rechentafel zum Stokes'schen Gesetz, *Koll. Z.*, 26 : 213–215 (1920).

[52] Tolman, R. C., and Vliet, E. B., A Tyndallmeter for the Examination of Disperse Systems, *J. Am. Chem. Soc.*, 41 : 297–300 (1919).

[53] Tolman, R. C., Reyerson, L. H., Vliet, E. B., Gerke, R. H., and Brooks, A. P., Relation between the Intensity of Tyndall Beam and Concentration of Suspensions and Smokes, *J. Am. Chem. Soc.*, 41 : 300–303 (1919).

[54] Wells, P. V., and Gerke, R. H., An Oscillation Method for Measuring the Size of Ultramicroscopic Particles, *J. Am. Chem. Soc.*, 41 : 312–329 (1919).

[55] Tolman, R. C., Gerke, R. H., Brooks, A. P., Herman, A. G., Mulliken, R. S., and Smyth, H. DeW., Relation between Intensity of Tyndall Beam and Size of Particles, *J. Am. Chem. Soc.*, 41 : 575–587 (1919).

[56] Tolman, R. C., Reyerson, L. H., Brooks, A. P., and Smyth, H. D., An Electrical Precipitator for Analyzing Smokes, *J. Am. Chem. Soc.*, 41 : 587–589 (1919).

is considered as having the same kinetic energy as a molecule. Accordingly, the fundamental equation,

$$PV = NRT \tag{16}$$

should hold for colloid systems. A gram molecule of a gas occupies approximately 22.4 liters volume at 0° and 760 mm. pressure. Inasmuch as the volume is inversely proportional to the pressure, a gram molecule of a gas would occupy 1 liter volume at 0° and approximately

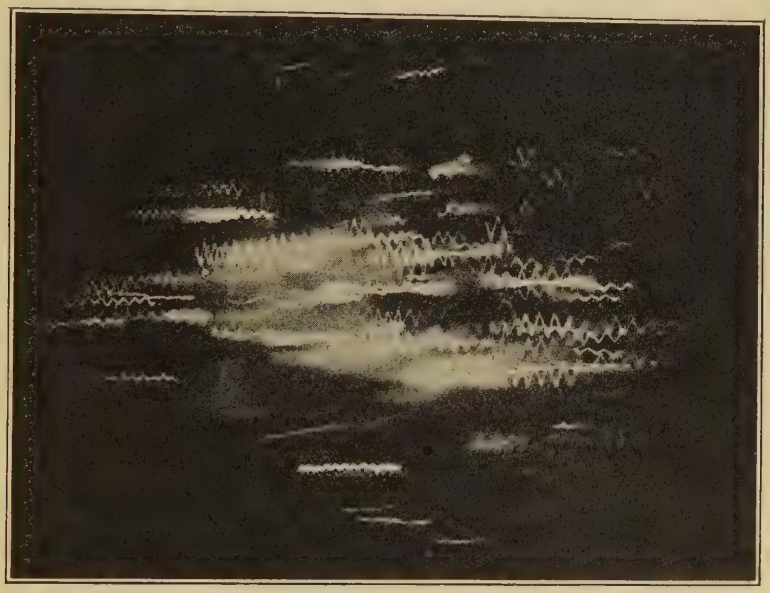


FIG. 19.—Ultramicroscopic photographs showing the vibration due to Brownian movement of colloidal particles moving across the field. Note difference in amplitude as indicating the presence of particles of different sizes. (Photograph, courtesy of Dr. P. V. Wells.)

22.4 atmospheres pressure. Solutions obey the gas laws provided that the volume in the above equation is the volume of the solvent and not the volume of the solvent plus solute. In a later section we will have occasion to discuss the osmotic pressure phenomenon exhibited by crystalloids and its relation to biological phenomena. At present we are only concerned with osmotic pressure exhibited by colloidal systems.

As noted in the section on Brownian movement, Perrin's gamboge sols had an apparent "molecular weight" of 30,000,000,000. Accordingly, 30,000,000 kilograms of the gamboge particles of the size studied by Perrin, if suspended in one liter of water, should give a sol possessing a normal osmotic pressure, *i.e.*, 22.4 atmospheres. Similarly, if a red

gold sol could be prepared containing approximately 300 pounds of red gold in one liter of water, we would observe an osmotic pressure of approximately 22.4 atmospheres. Red gold sols more concentrated than one gram of disperse phase in one liter of dispersions medium are rarely met with. Such sols as are obtainable show little or no osmotic pressure, and from the above considerations this is to be expected, inasmuch as a red gold sol containing only one gram of disperse phase in a liter of solvent should have an osmotic pressure only slightly greater than 0.1 mm. Hg, providing each particle possessed the kinetic energy of a molecule. Inasmuch as osmotic pressure is the driving force of diffusion, it is easy to understand why the disperse phase in most colloid systems possesses such a low diffusion coefficient.

The osmotic pressure of solutions of sols of bio-colloids, such as proteins, is extremely low even when the bio-colloid is present in mono-molecular condition. Sörensen[57] has made an elaborate study of the osmotic pressure of egg-albumin solutions. In Sörensen's studies of egg-albumin solutions he has definitely proven that the egg albumin is present in mono-molecular solution. As already noted in the earlier pages, this does not necessarily exclude egg-albumin solutions from being typical colloid sols. The author has no doubt but that they are typical colloidal sols, the micelles of which are individual egg-albumin molecules of a size sufficient (together with water of hydration) to bring the size of the molecule well within the limits of the colloidal realm. In Sörensen's studies of the osmotic pressure of egg-albumin sols, the pressure was measured against a water manometer. Directly measured osmotic pressures as high as 86 centimeters of water pressure were observed for an egg-albumin solution containing 22.66 grams of egg-albumin hydrate in 100 grams of water. Such values probably approach the upper limit of osmotic pressure values for lyophilic colloid systems. For most lyophilic systems much smaller values than these will be encountered, in many instances the osmotic pressure being only one or two centimeters of water pressure for similar concentrations, and for many lyophobic systems the value for osmotic pressure may be so small as not to be measurable.

Diffusion in Colloid Systems.—Graham's early distinction between colloidal and crystalloidal systems was largely based upon the pronounced difference in the rate of diffusion in the two instances. Graham found that if various materials were placed in bags of parchment paper or animal membrane, such as dried bladder or gold beater's skin, the various substances could be divided into two general classes:

[57] Sörensen, S. P. L., Studies on Proteins, Chap. V, *Compt. rend. trav. lab. Carlsberg*, Vol. 12, 1915–1917.

(1) those that readily passed through the parchment paper or animal membranes, and (2) those that failed to pass through such membranes. The former he called crystalloids, the latter colloids, and non-diffusion through a membrane was taken as the criterion of the colloid state.

The separation of colloids from crystalloids by the process of diffusion is called *dialysis*. Various membranes may be employed in dialysis. The more commonly used membranes are parchment paper, gold beater's skin, animal membranes and collodion membranes. Recently pure cellulose membranes in the form of cellophane sheets and tubes have replaced to a considerable extent collodion membranes for laboratory use.

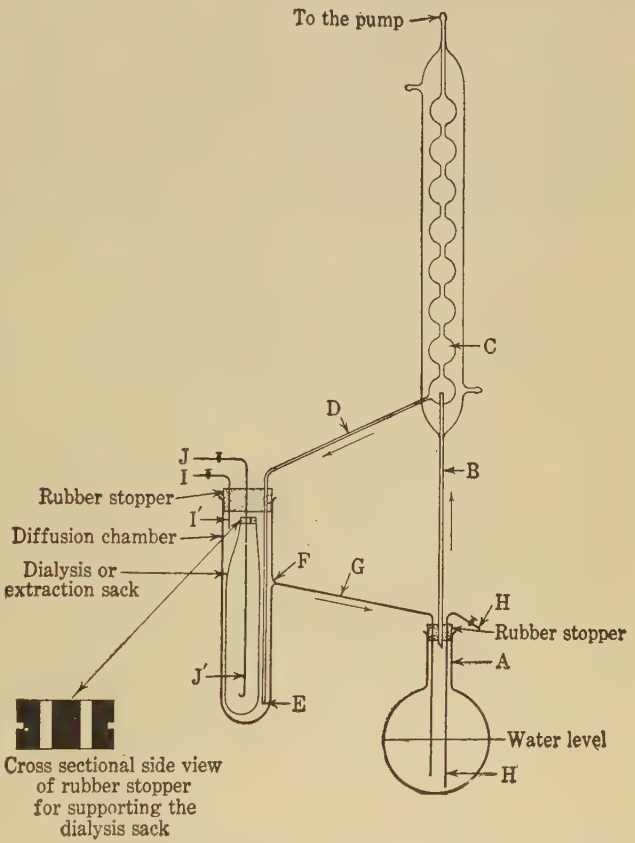


FIG. 20.—Apparatus for the continuous dialysis of biocolloids operating at reduced pressure with a constant volume of liquid. (After Hanke and Koessler.)

Hanke and Koessler[58] have devised an apparatus for continuous dialysis of hydrosols or hydrogels against water at constant volume. Such an apparatus is extremely useful in many biochemical operations, particularly where one desires to ascertain the ratio of colloid to total solids. Figure 20 shows the apparatus which Hanke and Koessler devised.

[58] Hanke, M. T., and Koessler, K. K., A Continuous Dialysis or Extraction Apparatus Which Operates at Reduced Pressure with a Constant Volume of Liquid, *J. Biol. Chem.*, 66 : 495–499 (1926).

It is obvious that if true solutions grade almost imperceptibly into colloidal systems and colloidal systems grade equally imperceptibly into coarse suspensions, there is no sharp line of demarcation, even with respect to rate of diffusion, between true solutions and colloid systems. We would accordingly expect to find true solutions which would have a low diffusion coefficient and pass extremely slowly through a dialyzing membrane, and we should likewise expect to find colloidal sols possessing a measurable rate of diffusion and likewise passing slowly through dialyzing membranes. It is therefore not surprising to find that egg albumin in appreciable amount may dialyze through a membrane. Membranes can, however, be prepared which are sufficiently dense to retain the egg-albumin molecules. Table VII lists certain more or less familiar materials with the corresponding coefficients of diffusion.

TABLE VII

Showing the Coefficients of Diffusion of Certain Common Crystalloidal and Colloidal Materials

	Coefficient of Diffusion	Temperature, ° C.
Nitric acid	2.10	20.0
Sodium chloride	1.04	20.0
Urea	0.81	7.5
Copper sulfate	0.47	17.0
Cane sugar	0.31	9.0
Nuclear gold sol (Svedberg)	0.27	11.7
Pepsin	0.070	18.0
Rennin	0.066	18.0
Egg albumin	0.059	18.0
Ovomucoid	0.044	18.0
Emulsin	0.036	18.0
Diphtheria toxin	0.014	12.0
Diphtheria antitoxin	0.0015	12.0
Tetanolysin	0.037	12.0
Antitetanolysin	0.0021	12.0

It will be noted that the coefficient of diffusion for sucrose lies very close to the coefficient of diffusion for the amicroscopic gold hydrosol. Accordingly, we should expect a very appreciable amount of amicroscopic gold hydrosol to pass through a membrane, and this is what actually takes place. On the other hand, the extremely low coefficient of diffusion for the anti-tetanolysin would mean that practically none of this material would diffuse, and this is what is actually observed. The dif-

fusion velocity is inversely proportional to the size of the particle, and an equation may be written,

$$\Delta r = K \tag{17}$$

where Δ = the diffusion coefficient;
r = the radius of the particle;
K = a constant.

According to this equation the diffusion velocities of two substances are inversely proportional to the radii of those substances, so that if we have one material, the diffusion coefficient of which is known, and we know the radius of the diffusing particles, we can determine either the diffusion coefficient or the radius of an unknown particle, providing one or the other is known, according to the equation,

$$\frac{\Delta_1}{\Delta_2} = \frac{r_2}{r_1} \tag{18}$$

Exner has shown that for gases

$$\Delta\sqrt{M} = K \tag{19}$$

where

M = the molecular weight,

and Öholm has shown that this holds for dilute solutions of non-electrolytes. We have already seen that colloid systems obey the gas laws, providing that each individual particle is regarded as a molecule. Accordingly, we can calculate the particle weight by equation (19), and it has been found that the particle weight, or so-called molecular weight, for egg albumin, hemoglobin, etc., calculated by this method, agrees fairly well with values obtained by other and independent physical measurements.

Svedberg[59] has used the equation

$$\Delta = \frac{RT}{N} \cdot \frac{1}{6\pi\eta r} \tag{20}$$

in order to test the behavior of colloids in relation to their kinetic energy. He found for a nuclear gold sol, $r = 1.29m\mu$. By an independent method not based on the diffusion velocity, a value of $r = 1.33m\mu$ was obtained. When the value of $1.33m\mu$ was taken as the value of r, and N was calculated from equation (20), an Avogadro constant of 58×10^{22} was obtained which agrees very well with the generally accepted values.

[59] Svedberg, The, Colloid Chemistry, Second Edition, p. 96, Chemical Catalog Company, Inc., New York (1928).

Certain special applications of phenomena involving diffusion in colloid gels will be taken up later.

Filtration and Ultrafiltration of Colloid Systems.—As a rule, colloid sols pass unchanged through filter paper or through the porcelain filters of the bacteriological laboratory. The pores in the usual filter paper are approximately 2–5μ in diameter, and the pores of the finer Berkefeld and Chamberlain porcelain filters range in size from 0.2–0.6μ. It is accordingly understandable why colloid sols should pass through such filters, inasmuch as the size of the colloidal particle is considerably less than the size of the opening through which the particle must pass. Matter in mass can, therefore, be separated from colloidal sols by passage through a bacterial filter.

Ultrafilters, however, have been devised with pores sufficiently fine to retain the disperse phase and allow only the dispersions medium and its crystalloidal solutes to pass through. This process of separation is known as ultrafiltration. Ultrafilters are invariably colloid gels, so that ultrafiltration may be defined as filtration of a sol through a gel.

Various types of ultrafilters have been devised. Those commonly used are membranes of collodion or gelatin, although rubber membranes have been used in some instances. The technic of ultrafiltration is largely due to the pioneer studies of Bechhold,[60, 61] who used filter paper and cloth as the supporting membrane for collodion or gelatin films. The paper or cloth was coated with collodion or with gelatin, the gelatin subsequently being hardened by immersion in a dilute solution of formaldehyde.

The direct measurement of osmotic pressure of crystalloidal solutions is carried out by making use of a membrane of copper ferricyanide precipitated in the pores of a porous porcelain vessel. Such a membrane can be made which is truly semi-permeable and which will allow the passage of water molecules but retard the passage of most crystalloidal solutions, such as solutions of dextrose, sucrose, etc. An ultrafilter differs only in degree from such a membrane, the pores of an ultrafilter being larger in diameter than the pores of a truly semi-permeable membrane. All gradations of pore size of ultrafilters can be obtained between the ranges of a copper ferricyanide membrane and ordinary filter paper, and numerous papers have been published, dealing with the technic for preparing such graded ultrafilters. One of the earlier papers is that by Schoep[62] who points out that almost any degree of permeability of col-

[60] Bechhold, H., Kolloidstudien mit der Filtrationsmethode, *Z. physik. Chem.*, 60 : 257–318 (1907).

[61] Bechhold, H., Durchlässigkeit von Ultrafiltern, *Z. physik. Chem.*, 64 : 328–342 (1908).

[62] Schoep, Alfred, Ueber ein neues Ultrafilter, *Koll. Z.*, 8 : 80–87 (1911).

lodion membranes can be obtained by mixing varying proportions of glycerol and castor oil with the collodion solutions. If it is desirable to conduct ultrafiltrations under high pressure, the membranes may be precipitated in a porous porcelain support. When so prepared, pressures up to 100 atmospheres may be applied to induce filtration.

Farmer[63] and Eggerth[64] have both studied the procedure for producing ultrafilters of varying porosity. Eggerth controlled porosity by varying the ratio of alcohol to ether in the solvent which was used to dissolve the collodion. He designated the porosity of the collodion membrane according to its " alcohol number," *i.e.*, the percentage of alcohol in the solvent used to dissolve the collodion. Figure 21 shows the relationship which he found between alcohol number and the diameter of

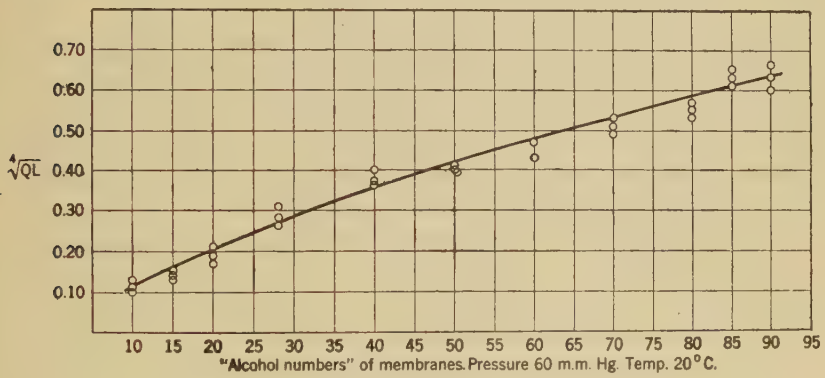


FIG. 21.—Showing the relation between permeability of ultrafilters and Eggerth's "alcohol numbers." (Data of Eggerth.)

the pores. He points out that the diameter is proportional to $\sqrt[4]{QL}$, where Q is the volume of liquid passing through the filter in unit time, and L is the thickness of the membrane.

Duclaux and Errera[65] studied the mechanism of ultrafiltration and point out that the velocity of flow of liquid through the pores of the membrane is, as was to be expected, inversely proportional to the viscosity of the liquid and directly proportional to the pressure, the membrane behaving exactly like a bundle of fine capillary tubes. On the other

[63] Farmer, C. J., A Method for the Preparation of Uniform Collodion Membranes for Dialysis, *J. Biol. Chem.*, 32 : 447–453 (1917).

[64] Eggerth, A. H., The Preparation and Standardization of Collodion Membranes, *J. Biol. Chem.*, 48 : 203–221 (1921).

[65] Duclaux, J., and Errera, J., Le mécanisme de l'ultra-filtration, *Rev. Gen. Colloides*, 2 : 130–139 (1924).

hand, Brinkman and Szent-Györgyi[66] point out that surface tension or interfacial tension may profoundly alter the characteristics of a collodion ultrafilter. They found that when a hemoglobin sol was placed in a collodion bag and subjected to a pressure of 3 atmopsheres, the hemoglobin was completely retained and only water passed the membrane. On the other hand, if a dilute solution of sodium oleate was first passed through the filter, the membrane became permeable to the hemoglobin. The hemoglobin which passed the sodium oleate-treated membrane would not subsequently pass through a second untreated membrane. Accordingly, the size of the micelles in the hemoglobin sol which had passed through the treated membrane had not been affected by the sodium oleate. The size of the pores in the treated membrane had not been altered, as was shown by rate of water filtration through the pores before and after the sodium oleate treatment. Sodium caproate, which has little or no effect on interfacial tension, did not alter the properties of the collodion membrane. Sodium linoleate, sodium glycocholate, digitonin, α-mono-olein-glycerol ester and Witte's peptone, which lower the interfacial tension in varying degrees, affect the permeability of the collodion membrane in the same ratio as they affect interfacial tension.

In a second paper[67] they studied the effects of alkaloids and purine bases on permeability of ultrafilters, using atropine, pilocarpine, caffeine, strychnine, quinine, and morphine. A change in the permeability of the collodion membrane toward hemoglobin was noted. Biologically inactive codeine did not alter the permeability; neither did cocaine nor novococaine. These results are very striking, and, as Brinkman and Szent-Györgyi suggest, may have a fundamental bearing on reactions which take place in living organisms.

Lundsgaard and Holbøll[68] have likewise studied the preparation, standardization, and calibration of collodion membranes. They use a rather novel method of determining porosity. A glucose solution of known concentration is placed in a collodion bag or tube and allowed to dialyze against pure water for a definite interval of time. Analysis for

[66] Brinkman, R., and Szent–Györgyi, A. v., Studien über die physikalisch-chemischen Grundlagen der vitalen Permeabilität. I. Die Wirkung kapillaraktiver Stoffe auf die Permeabilität von Kollodiummembranen, *Biochem. Z.*, 139 : 261–269 (1923).

[67] Brinkman, R., and Szent–Györgyi, A. v., Studien über die physikalisch-chemischen Grundlagen der vitalen Permeabilität. II. Die Wirkung von Alkaloiden und Purinbasen auf die Permeabilität von Kollodiummembranen, *Biochem. Z.*, 139 : 270–273 (1923).

[68] Lundsgaard, C., and Holbøll, S. A., Investigations into and the Standardization and Calibration of Collodion Membranes I., *J. Biol. Chem.*, 68 : 439–456 (1926).

glucose in the inner and outer liquids is made at intervals. When the volume of liquid inside the membrane is equal to the volume of liquid outside the membrane, the following equation can be applied

$$\frac{dx}{dt} = \Delta\left(\frac{K}{2} - x\right) \tag{21}$$

where K = the original concentration of glucose in the inner liquid;
x = the increase in glucose concentration in the outer liquid in time, t;
Δ = diffusion coefficient of glucose for a given membrane.

Accordingly,

$$\Delta = \frac{1}{t}\log\frac{K}{K - 2x} \tag{22}$$

Lundsgaard and Holbøll found from experiment that the original concentration of glucose (within limits ranging from 0.1 per cent to 0.3 per cent concentration) made no difference in the value obtained for the diffusion coefficient. They prepared membranes of definite porosity by coating glass tubes with collodion and then immersing the collodion membrane, when partially dry, in alcohol of known concentration for a fixed period of time, followed by immersion in water. When alcohol of 70 per cent concentration was employed, the diffusion coefficient of glucose through the membrane ranged from $\Delta = 0.0133$ to $\Delta = 0.0122$ as maximum and minimum ranges (eight membranes tested). When 80 per cent alcohol was used, the maximum and minimum ranges were $\Delta = 0.0097$ to $\Delta = 0.0093$, and when 90 per cent alcohol was used, similar values were $\Delta = 0.0058$ to $\Delta = 0.0057$. They state that the diffusion coefficient is not altered after the membranes have been aged for one week. Hitchcock[69] has applied the viscosity formula to the measurement of the size of pores in collodion membranes. In the membranes that he worked with, the radius of the pores ranged from $20.8m\mu$ to $2.7m\mu$. He points out that in the former case there are 70 billion capillary tubes per square centimeter and in the latter case 2700 billion capillary tubes per square centimeter.

Bechhold and Heymann[70] have used ultrafiltration to concentrate gelatin sols, the dispersions medium passing through and the gelatin micelles being retained on the filter. In this way the ash content was greatly reduced. Using membranes of varying porosity they state that

[69] Hitchcock, D. I., The Size of Pores in Collodion Membranes, *J. Gen. Physiol.*, 9 : 755–762 (1926).

[70] Bechhold, H., and Heymann, E., Konzentrierung und Reinigung von Lösungen hydrophiler Kolloide, *Biochem. Z.*, 171 : 33–39 (1926).

they were able to separate gelatin into two fractions, one of which passed through the more porous membrane.

It is often desirable to test the porosity of a membrane by more or less qualitative methods. This can be done by making use of a series of colloidal sols ranging from a Prussian blue sol to a truly crystalloidal solution. The following list of materials has been suggested by various workers as offering a graded series of particle size.

Prussian blue > Bredig's platinum sol > casein in milk > As_2S_3 sol > Zsigmondy's red gold sol > 1 per cent gelatin sol > hemoglobin sol > litmus > nuclear gold sol > crystalloids.

It is sometimes impossible to use ultrafiltration procedure, due to the fact that collodion or gelatin membranes are altered by the liquid which one desires to filter. Since such membranes are colloid gels, they would be affected by hot solutions and by solutions which are definitely acid or alkaline. Manning[71] has prepared metallic ultrafilters by plating nickel on phosphobronze or nickel gauze under such conditions that the plated nickel is deposited in a porous state. His paper gives definite directions for current density and concentration of solutions which should be employed. He points out that such ultrafilters can be used with organic solvents and that they are not affected by relatively high temperatures.

Perhaps the most striking contribution to the recent literature of ultrafiltration is the observation by Kramer,[72] who emphasizes that the nature of the charge on the colloid membrane had not been taken into account in previous studies of filtration. Kramer was interested particularly in the nature of the so-called filterable micro-organisms and viruses which pass unaltered through the porcelain filters of the bacteriological laboratory. He points out that all Berkefeld and Pasteur-Chamberlain filters are made from siliceous materials and consequently possess a negative charge. In testing such filters, he found that colloidal dyestuffs possessing a positive charge would not pass such filters, whereas those which were negatively charged passed readily. For example, Victoria blue (+) was retained in a Berkefeld filter, whereas Congo red (−) readily passed through. He, accordingly, attempted to prepare filters similar to the Berkefeld filter but possessing a positive charge. Using plaster of Paris ($CaSO_4$) filters, he found no differentiation between acid and basic dyestuffs. When, however, a small amount (± 5 per cent) of calcium carbonate was added to the plaster of Paris prior to

[71] Manning, John, The Preparation of Nickel Membranes for Ultrafiltration, *J. Chem. Soc.* (*London*), May, 1926, 1127–1132.

[72] Kramer, S. P., Bacterial Filters, *J. Gen. Physiol.*, 9 : 811–812 (1926).

forming the filter, he obtained positively charged filters which retained the Congo red (−) sols and allowed the Victoria blue (+) to pass through, thus reversing the retainability of the Berkefeld filter. A still more striking observation of Kramer is the fact that the bacteriophage of *Staphylococcus aureus*, the *Vibrio percolans* of Mudd, vaccine virus, and rabies virus are retained by the positively charged plaster of Paris filters, whereas they readily pass through the ordinary bacteriological filters. As we shall see later in a discussion of the electrical properties of colloids, the retention is probably due to the mutual precipitation of

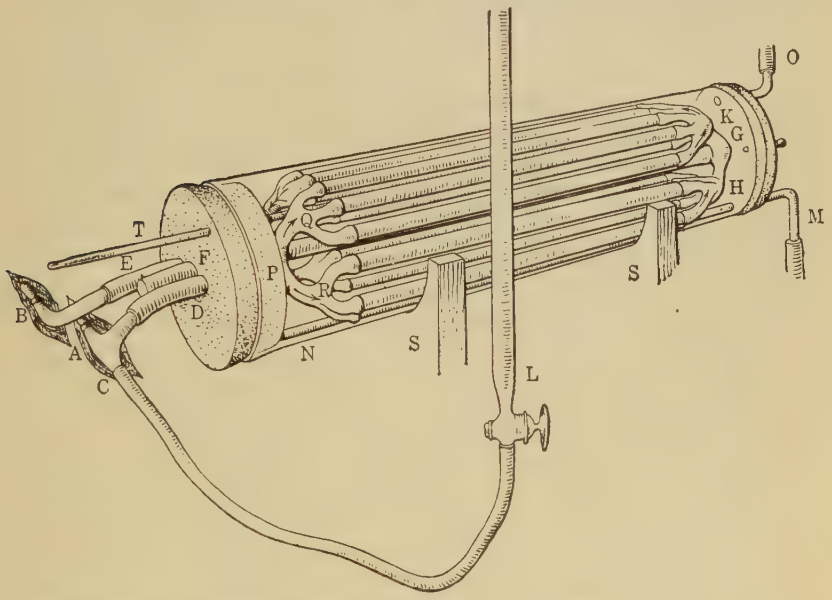


FIG. 22.—Abel's vividiffusion apparatus. Vein at *B*, artery at *C*, anticoagulant added at *L*.

oppositely charged micelles. Kramer's observations should open a new field in the study of virus problems.

VIVIDIFFUSION.—Abel, Rowntree, and Turner[73] have applied the methods of dialysis and ultrafiltration to a study of the crystalloids present in the blood stream. Figure 22 shows the apparatus which they devised. Using a glass cylinder open at both ends, they inserted within the cylinder a number of collodion tubes, each 8 mm. in diameter and 20 cm. in length. These tubes were connected in series by glass

[73] Abel, J. J., Rowntree, L. G., and Turner, B. B., On the Removal of Diffusible Substances from the Circulating Blood of Living Animals by Dialysis, *J. Pharm. Exp. Therap.*, 5 : 275–316 (1914).

U-tubes, together with in-flow and out-flow tubes which passed through rubber stoppers closing the ends of the glass cylinder. With the tubes in place and filled with physiological salt solution and surrounded with physiological salt solution, the in-flow tube was connected to the carotid artery of the experimental animal, the out-flow tube being connected to the femoral vein. Hirudin (leach extract, an anti-blood-coagulant) is injected into the blood before it passes from the carotid artery into the system of collodion tubes, thus preventing the blood from coagulating when it comes in contact with the glass connecting tubes. The liquid surrounding the collodion tubes is kept at blood temperature by means of a thermostat. Thus, it is possible to pass blood from the heart of an animal outside of the body through a dialyzing and ultrafiltration apparatus, back through the venous circulation into the animal body, and continue this cycle of blood circulation over relatively long periods of time.

The liquid on the outside of the collodion tubes may be a physiological salt solution or it may be distilled water. In the latter case, there is, of course, a rapid loss of the inorganic constituents of the blood. As we shall see later in a discussion of osmotic phenomena, a crystalloid tends to collect in equal concentrations both within and without a membrane to which it is permeable. Accordingly the crystalloidal constituents of the blood pass through the collodion membrane into the outer liquid which can be drawn off at intervals and replaced with fresh liquid. In this way the crystalloidal constituents can be separated from the colloidal constituents of the blood.

Abel and his coworkers have constructed various vividiffusion cells, the number of collodion tubes ranging from 2 to 32 or more. In some instances vividiffusion has been kept up continuously for a period in excess of ten hours. In preliminary experiments 1 gram of sodium salicylate was slowly injected into the femoral vein of a dog weighing 7 kilograms. Of the total amount of salicylic acid injected, 19.1 per cent was recovered in the diffusion liquid of the vividiffusion apparatus, whereas only 17.5 per cent was eliminated in the urine during the same period of time, thus showing that the apparatus can compete with the kidneys on favorable terms, at least during a part of the dialysis period.

This apparatus gave definite proof that amino acids circulated in the blood stream in the free state. Abel demonstrated that there was a marked increase in amino acids in the diffusion liquid following the feeding of a meat diet to a dog which was being used for the vividiffusion experiment. This was apparently the first demonstration that proteins on digestion passed into the blood in the form of free amino acids and were carried in this form from the blood to the various cells and tissues of the body.

In a later paper the authors[74] report on the chemical composition of the material which had diffused through the collodion membranes. In a series of vividiffusion experiments, covering a total of 112 hours, nitrogenous compounds, containing a total of 20 grams of nitrogen, were obtained. A large part of this nitrogen was, as would be expected, urea nitrogen. However, histidine, isobutyl-hydantoin, alanine, valine, lactic acid, β-oxy-butyric acid, and creatinine were identified. Other substances, especially glucose, dialyzed in large quantities. The quantities of the materials which were identified accounted for only a fraction of the total solids which were obtained. Abel notes that the dialysates contained considerable quantities of materials which did not yield to ready identification.

Abel regards the vividiffusion apparatus as similar to an artificial kidney and has suggested that it might be used in case of severe poisoning in order to remove the poisonous elements from the blood stream. Its value in such connections would, of course, depend upon the length of time which had elapsed between the taking of the poison and the beginning of the vividiffusion process. This apparatus should be a valuable tool in physiological studies. Abel notes that with proper technic the blood passing through an individual organ could be passed through a vividiffusion apparatus. In this way the diffusible constituents which any individual organ contributes to the blood stream could be studied.

Electrodialysis.—Dialysis which depends only upon diffusion is often an extremely slow process, and it is sometimes impossible to remove the last traces of adsorbed electrolytes. The removal of electrolytes from colloid sols and gels may be hastened and the colloidal material obtained in a much higher degree of purity by dialyzing with the aid of an electric current. Electrolytes in aqueous solutions are dissociated into ions, and these ions move under the influence of a direct current to the anode and the cathode. If the colloidal sol is restrained by suitable membranes, from moving to the anode and cathode compartments, electrolytes can be then almost completely removed. Sheppard, Sweet, and Benedict[75] used this method to prepare ash-free gelatin. Electrodialysis was carried out in a large wooden cell, in the middle of which was placed a porous inner compartment of silica which extended to and was imperviously cemented to both the bottom and the sides of the

[74] Abel, J. J., Rowntree, L. G., and Turner, B. B., On the Removal of Diffusible Substances from the Circulating Blood of Living Animals by Dialysis II. Some Constituents of the Blood, *J. Pharm. Exp. Therap.*, 5 : 611–623 (1914).

[75] Sheppard, S. E., Sweet, S. S., and Benedict, A. J., Elasticity of Purified Gelatin Jellies as a Function of Hydrogen-ion Concentration, *J. Am. Chem. Soc.*, 44 : 1857–1866 (1922).

wooden cell. The silica container was filled with 5 per cent gelatin, the two end compartments of the wooden cell were filled with distilled water, and the whole electrodialyzed, using 110 volts, direct current, and a gold anode and a silver cathode. By this procedure gelatin containing from 0.02 to 0.03 per cent ash was obtained.

Knaggs, Manning, and Schryver[76] electrodialyzed gelatin by allowing a 10 to 20 per cent gelatin gel to set in the lower part of a bell jar, suspending this in water over a mercury cathode and inserting a platinum anode in water above the gelatin gel, as is shown in Fig. 23. The electrolysis was conducted with 100 to 220 volts, direct current, the water being changed periodically, and the process being continued until

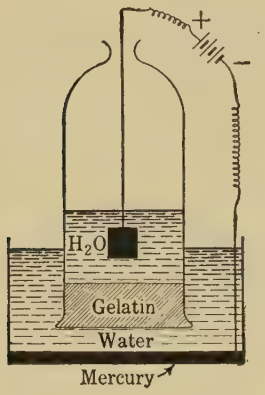


Fig. 23.—Electrodialysis of gelatin as suggested by Knaggs and Schryver.

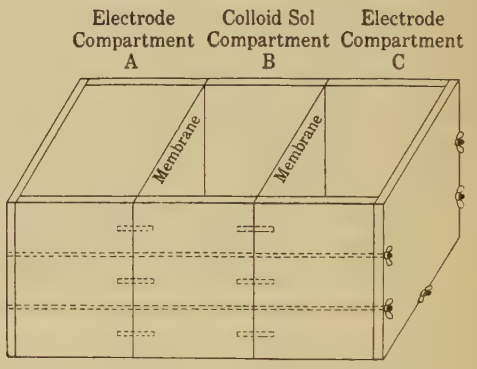


Fig. 24.—Wooden box suitable for electrodialysis.

there was no further appearance of alkali at the cathode or acid at the anode. The ash content in this way was reduced to 0.02 per cent or less.

Hoffman and Gortner[77] used electrodialysis to study the composition of agar. They constructed a wooden box, heavily impregnated with paraffin, which had previously been sawed into three sections and which could again be fitted together by dowels extending from one section into holes bored in the other sections. Figure 24 illustrates such an apparatus. The membranes separating sections (*A*) and (*B*), and sections (*B*) and (*C*) may be either collodion membranes, cellophane

[76] Knaggs, J., Manning, A. B., and Schryver, S. B., Investigations on Gelatin, Part II. Researches on the Methods of Purifying Gelatin, *Biochem. J.*, 17 : 473–487 (1923).

[77] Hoffman, W. F., and Gortner, R. A., The Electrodialysis of Agar. A Method for the Preparation of the Free Agar-Acid, *J. Biol. Chem.*, 65 : 371–379 (1925).

membranes, parchment paper, or animal membranes of various kinds. The electrodes are inserted in compartments (*A*) and (*C*). The colloid sol, which it is desired to purify, is placed in the center compartment (*B*). Gold or platinum electrodes are preferable, although graphite plates have been used with considerable satisfaction. Compartments (*A*) and (*C*) are filled with distilled water, the electrodes connected to a source of direct current are inserted, and electrolysis continued, changing the water in the anode and cathode compartments at frequent intervals until the conductivity of the system is reduced to approximately zero and until tests of the liquid in the anode compartments show complete absence of anions, such as chloride, sulfate, phosphate, etc., and the liquid in the cathode compartment shows the absence of metallic ions.

Using this apparatus, they were able to show that the sulfur content of agar is organically bound in the molecule in the form of a substituted sulfuric acid, whereas the agar was freed from other inorganic elements. As usually obtained, agar is neutral in reaction. Electrodialyzed agar, on the other hand, is a strong acid, a 1 per cent solution having a *p*H of approximately 2.5. The silica which was present in the original agar did not pass through the membrane, indicating that it probably was in colloidal form.

Recently Thomas and Murray[78] have electrodialyzed gum acacia, and have prepared an arabic acid, a 1 per cent solution of which had a *p*H value of 2.70. They found, however, that arabic acid was not a sulfuric acid ester, as is agar acid.

As already noted, it is frequently impossible to completely free colloid sols from electrolytes by simple dialysis. Electrolytes are frequently adsorbed on the surface of the colloid micelles and are held there by electrical forces. Under such conditions, the electrolytes become to all intents and purposes a part of the colloidal micelle. It is only by the use of an impressed electrical potential that the ions can be removed from the surfaces of the colloidal particles. In this way electrodialysis makes not only for an increased rapidity of the purification process but likewise for greater purification.

Electro-ultrafiltration.—Bechhold and Rosenberg[79] have suggested a direct electric current as an aid to ultrafiltration and have applied this technic to the purification of gelatin. Figure 25 illustrates diagrammatically their apparatus. On the bottom of a Büchner funnel is placed a perforated metallic disc which forms the cathode. On top of

[78] Thomas, A. W., and Murray, H. A., A Physico-Chemical Study of Gum Arabic, *J. Phys. Chem.*, 32 : 676–697 (1928).

[79] Bechhold, H., and Rosenberg, A., Elektro-Ultrafiltration von Gelatine und Leim, *Biochem. Z.*, 157 : 85–97 (1925).

this perforated metallic disc is placed the membrane of the ultrafilter. The liquid to be filtered is then placed above the ultrafilter in the Büchner funnel, and the anode is inserted in liquid in a collodion bag which is suspended in the colloid sol in the Büchner funnel. The anions, present as contaminations in the sol, migrate through the collodion bag to the anode and can be removed by drawing off the liquid in the collodion bag at frequent intervals. The cations pass through the ultrafilter and are washed away in the water which passes through the ultrafilter. An additional phenomenon, known as electroendosmosis, hastens the ultrafiltration. Inasmuch as the ultrafilters are generally negatively charged, water is drawn by the electric current through the ultrafilter to the pole having the same sign as the electrical charge on the ultrafilter. The principle of electroendosmosis will be discussed later. Bechhold and Rosenberg find, however, that using apparatus similar to that shown in Fig. 25, they are able to very rapidly ultrafilter colloidal sols at either atmospheric pressure or slightly reduced pressures, and that electro-ultrafiltration is a much more rapid process than the usual ultrafiltration methods, and that colloidal sols of much higher purity can be obtained. At the same time the phenomenon of electroendosmosis affords a means by which the colloidal sol can be greatly concentrated. Using this technic. they report the separation of gelatin into two protein fractions.

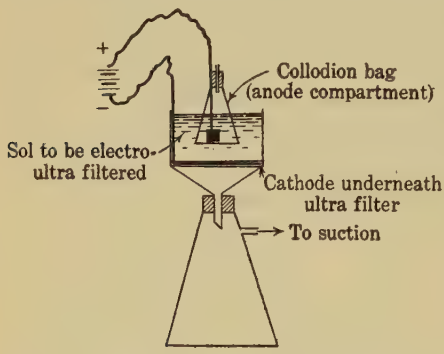


FIG. 25.—Combined electrodialysis, and electro-ultrafiltration. (After Bechhold.)

OPTICAL PROPERTIES OF COLLOID SYSTEMS.—Everyone has observed motes of dust floating in the air when illuminated by a beam of sunlight or in the rays of a projection lantern. In such cases the visibility of the dust particles is due to the fact that they act more or less as mirrors reflecting the light rays at an angle so that the observer sees in reality the light source rather than the actual surface of the dust particle. Tyndall[80] was the first to investigate this phenomenon. The light which impinges on a particle is not polarized, whereas the light reflected

[80] Tyndall, J., On the Blue Colour of the Sky, the Polarization of Skylight, and on the Polarization of Light by Cloudy Matter Generally, *Phil. Mag.*, (4) 37 : 384-394 (1869).

from the particle is generally strongly polarized. Faraday[81] in exhibiting his red gold sols noted that, " when a light is looked at through the fluid, the latter appears transparent; but when the eye is on the illuminated side, then the fluid is seen opalescent. If a cone of sun's rays be thrown by a lens into the fluid, the illumination of the particles within the cone shows their presence as undissolved bodies." This phenomenon has become known as the *Tyndall phenomenon* and the illuminated path of suspended particles as a *Tyndall cone.*

Colloid systems differ greatly in the intensity with which they show the Tyndall cone. Lyophobic colloids, as a rule, show intense Tyndall cones. Lyophilic colloids, on the other hand, may either show no Tyndall cone or only a faint Tyndall cone. The degree to which a Tyndall cone is shown depends upon the difference between the index of refraction of the dispersions medium and of the disperse phase. If there is a large difference in index of refraction between the two phases, a strong Tyndall cone will result. If there is little or no difference in index of refraction, a faint or no Tyndall cone will be observed. Accordingly, one cannot be certain from the appearance of the Tyndall cone whether one is dealing with a lyophilic or a lyophobic sol. Glass would form a lyophobic sol both in water and in ethyl iodide. The hydrosol would provide a considerable difference in refractive index between the phases and a strong Tyndall cone would result. On the other hand, the index of refraction between the phases in the organosol would be very slight and only a faint Tyndall cone should result. It should be possible to secure samples of glass having the same refractive index as the ethyl iodide, in which case the lyophobic sol would be optically clear.

Lyophilic sols range from those which show a weak Tyndall cone to those which are practically optically clear. The reason for their transparency lies in the fact that they are highly solvated. A part of the dispersions medium is associated with the disperse phase; the disperse phase is greatly swollen by the dispersions medium which has dissolved in it, and accordingly its index of refraction has been brought very close to the index of refraction of the pure dispersions medium. There is therefore very little light refraction from the surface of the particles.

A jelly fish may contain as much as 98 per cent of water in the living condition. When such an organism is floating in the sea, it is in many instances practically invisible, due to the fact that the rays of light pass nearly equally well through the water and through the organism. When it is removed from the water, it is, of course, plainly visible, due to the difference in refractive index between the air and the organism.

[81] Faraday, M., On the Relations of Gold and Other Metals to Light, *Proc. Roy. Soc.*, 8 : 356–361 (1857).

As already noted, Tolman and Vliet[82] have devised a Tyndallmeter for the examination of disperse systems. A cloud of colloidal particles or a fine precipitate which is invisible in the ordinary room may be easily visible when a powerful beam of light is focused on the system. Thus, for example, in titrating a potassium cyanide solution with silver nitrate, it is possible by conducting the titration in a strong beam of light to detect the end point where the precipitate of silver cyanide begins to form, some time before it is visible in the absence of the beam. Accordingly a much sharper titration and a much more accurate titration can be made by watching for the first appearance of the Tyndall cone in the solution.

The principle involved in the Tyndall cone is used in analytical chemistry under the name of nephelometry. Kober[83,84] and Kober and Graves[85] have adequately discussed the principles which are involved. Nephelometric analysis depends upon the refraction of light from the surface of a precipitate and is used where the precipitate is too small in amount for accurate gravimetric determination and where it possesses no color, so that colorimetric methods are not applicable. In the determination of atomic weights, where either silver or chlorine is used as the reference standard, the amount of silver chloride remaining in the wash water and mother liquors is usually accounted for by nephelometric analysis.

When light is reflected from a surface, the short, rapid light rays are usually bent more than are the long, slow rays. Accordingly, the blue, violet, and ultraviolet rays are bent more than the red and yellow rays, and in many instances a partial separation of the spectrum results. This is known as opalescence. A sol containing suspended colorless particles may appear to be pale blue. Various sized particles of the same material may affect the distribution of the reflected rays in different manners. Thus, for example, colloidal gold sols may be colored orange, red, lavender, violet, indigo blue, or black, depending upon the size of the particles of gold in the hydrosol, the smaller sized particles being at the orange end of the list, the larger sized ones appearing deep blue or black. Herzfeld

[82] Tolman, R. C., and Vliet, E. B., A Tyndallmeter for the Examination of Disperse Systems, *J. Am. Chem. Soc.*, 41 : 297–300 (1919).

[83] Kober, P. A., An Improved Nephelometer-Colorimeter, *J. Biol. Chem.*, 29 : 155–168 (1917).

[84] Kober, P. A., Technical Applications of Nephelometry, *J. Ind. Eng. Chem.*, 10 : 556–563 (1918).

[85] Kober, P. A., and Graves, S. S., Nephelometry (Photometric Analysis) I. History of Method and Development of Instruments, *J. Ind. Eng. Chem.*, 7 : 843–847 (1915).

and Klinger[86,87] have suggested that in all probability the colors produced when iodine is added to starch or dextrin sols are in reality only manifestations of the size of the colloidal micelles and the different colors do not necessarily indicate chemical differences.

The human eye is sensitive to only a narrow band of radiant energy. Accordingly, the Tyndall cones which we actually see are derived from only that portion of the light to which the retina is sensitive. When ultraviolet light is used as a source of illumination we are unable in many instances to see the Tyndall cone which results. However, many systems which are optically empty to the eye may show a marked Tyndall cone when photographed under ultraviolet light. Certain lyophilic systems, which are optically empty with ordinary light, show markedly visible Tyndall cones when a beam of ultraviolet light is projected into them. This phenomenon is known as *fluorescence*, the ultraviolet light being transformed into longer wave lengths which are visible to the eye. Protein sols and gels in particular show such fluorescence. Svedberg and Tiselius[88] have used the fluorescence of proteins in order to render visible the boundary between an egg albumin sol and the surface of a solution which does not contain protein.

Since a photographic plate may show the presence of a Tyndall cone in molecularly disperse systems, it is obvious that if shorter wave lengths were employed, light refraction would occur from the surface of individual molecules or ions. This is actually the case. When X-rays are employed they are refracted from the surface of individual atoms within crystals so as to give the familiar spatial orientation of atoms from which so much of our recent knowledge of crystal structure has been derived.

The following diagram illustrates certain of the optical properties of systems having various degrees of dispersion:

Atoms	Molecularly Dispersed Systems	Colloidal Systems	Suspensions	
	Amicrons	Ultramicrons	Microns	
Tyndall phenomenon by Röntgen rays	Tyndall phenomenon by ultraviolet rays	Tyndall phenomenon by visible light rays	Turbid appearance	Coarse particles visible

[86] Herzfeld, E., and Klinger, R., Zur Chemie der Polysaccharide, *Biochem. Z.*, 107 : 268–294 (1920).

[87] Herzfeld, E., and Klinger, R., Berichtung und Ergänzung zu unserer Arbeit: "Zur Chemie der Polysaccharide," *Biochem. Z.*, 112 : 55–60 (1920).

[88] Svedberg, The, and Tiselius, A., A New Method for Determination of the Mobility of Proteins, *J. Am. Chem. Soc.*, 48 : 2272–2278 (1926).

While considerable qualititative information in regard to colloid systems can be obtained by use of the Tyndall beam, it is not directly applicable to a careful study of colloid systems. However, Siedentopf and Zsigmondy[89] have devised an instrument known as the ultramicroscope, making use of the general principles of the Tyndall phenomenon. If a powerful beam of light is focused to a point within a colloid sol and if the rays are refracted from the surface of the disperse phase, one may view the position and the Brownian movement of individual particles through a microscope. Figure 26 shows diagrammatically the path of such light rays from a light source to a colloid particle through a lens to the observer's eye.

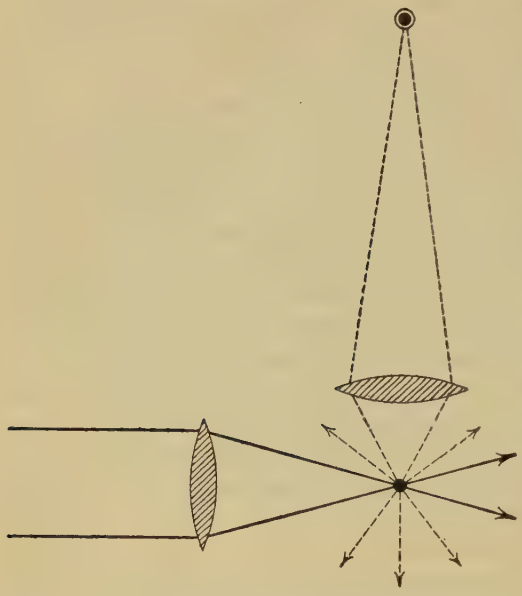


FIG. 26.—The diffraction of light impinging upon an ultramicroscopic particle, the axes of the illuminating and diffracted rays being at right angles to each other. (Solid lines, illuminating rays; dotted lines, diffracted rays.)

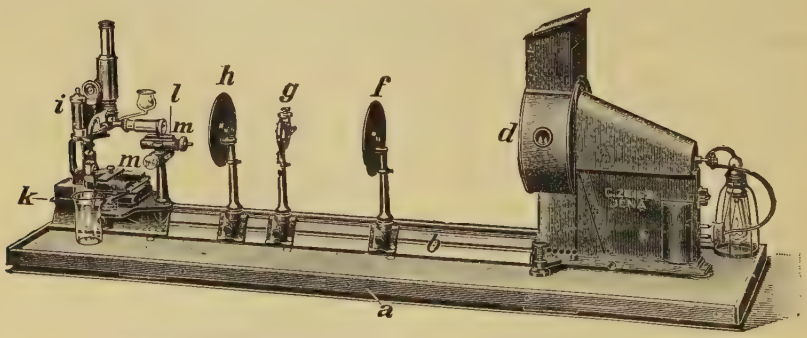


FIG. 27.—The Siedentopf–Zsigmondy slit ultramicroscope.

[89] Siedentopf, H., and Zsigmondy, R., Über Sichtbarmachung und Grössenbestimmung ultramikroskopischer Teilchen, mit besonderer Anwendung auf Goldrubingläser, *Ann. d. Physik* (4) 10 : 1–39 (1903).

Figure 27 shows the arrangement of the Siedentopf-Zsigmondy slit ultramicroscope for the examination of ultramicroscopic particles. The principle is the same as the technic sometimes used in signaling in warfare and boy scout activities, and known as heliography. One person on a hilltop, perhaps several miles away, holds in his hand a small mirror about two or three inches in diameter; holding this mirror so as to catch the sun's rays, the rays are flashed in the Morse code across the intervening valley to the receiver stationed at a distance. The receiver does not observe the mirror which itself is far too small to be seen with the unaided eye across the intervening distance, but rather he sees the light source, the sun, reflected from the surface of the mirror. In the same way the observer, looking into an ultramicroscope, does not see the colloid particle. The particle is too small to be seen with the magnification employed, so that the individual bright particles which are viewed in the ultramicroscope do not indicate either the form or the size of the ultramicroscopic particle but instead they merely act as mirrors, and the observer sees reflected from the surface of such a mirror the source of light which is used for illumination, perhaps a carbon arc or for more intense illumination the direct image of the sun.

Fig. 28.—Photomicrograph of the gold particles in a red gold sol as viewed with a Siedentopf-Zsigmondy slit ultramicroscope.

Figure 28 is a photomicrograph of a Zsigmondy red gold sol taken as viewed through a Siedentopf-Zsigmondy slit ultramicroscope. In this particular sol an amount of auric chloride equivalent to 6 milligrams of metallic gold was dissolved in 500 cc. of water and reduced to the colloidal metal by the action of formaldehyde. One cc. of this brilliantly red hydrosol was then removed and diluted to 500 cc., and it is

this dilute hydrosol which was used for the photomicrograph. In other words, the system which was photographed contained only 0.012 mg. of gold in 500 cc. volume. Nevertheless this 0.012 mg. of gold was sufficient to form approximately 625,000,000,000 colloid particles.

The ultramicroscope field which is shown in Fig. 28 is still far too concentrated for quantitative studies using the ultramicroscope, but it illustrates the point which it is desired to emphazise, *i.e.*, the immense number of colloid particles that may result from a relatively small weight of material.

A gold hydrosol is particularly adapted to ultramicroscopic studies because of the completeness with which light is reflected from a gold surface. If an egg-albumin sol or the hypothetical glass-ethyl iodide sol already noted were examined under the ultramicroscope, the field would be practically empty. Accordingly the test of ultramicroscopic visibility is not necessarily a valid criterion as to whether a given disperse phase is lyophilic or lyophobic, and the absence of ultramicrons in the ultramicroscope, contrary to the argument of McClendon and Prendergast,[90] is no proof that truly colloidal micelles are not present.

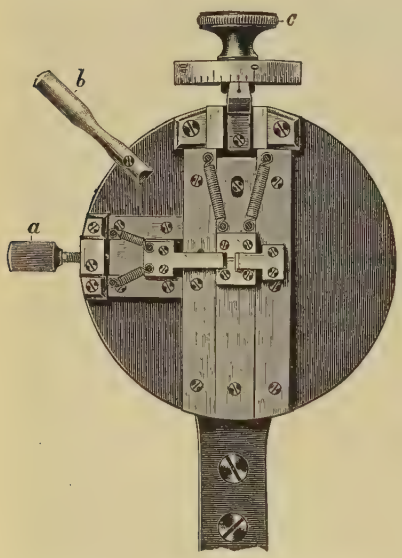


FIG. 29.—Adjustable slit for the Siedentopf-Zsigmondy ultramicroscope.

Referring again to Fig. 27, the beam from the electric arc in the lamp-housing, (*d*), passes through the lens, (*f*), which concentrates the beam and throws it on an adjustable slit, (*g*). The arrangement of this adjustable slit is shown in Fig. 29. It will be noted that the slit can be adjusted by the micrometer screws, (*a*) and (*c*), the latter having attached to it a graduated drum reading in microns. Accordingly, the width of the slit in one direction can be accurately known. Lever (*b*), Fig. 29, affords a means by which the adjustable slit may be rotated at right angles, so that the slit of known dimensions can be altered from depth to breadth in the solution. In this way an area of known dimensions can be illuminated, the number of particles in this known area can be counted, and accordingly the concentration of ultramicroscopic par-

[90] McClendon, J. F., and Prendergast, H. J., Note on the Ultramicroscopy of Egg Albumin, *J. Biol. Chem.*, 38 : 549 (1919).

ticles per cubic centimeter can be ascertained. As the beam of light passes through slit, (*g*), it again passes through a second lens, (*h*), and into a microscope objective, (*l*), from which it emerges and passes through a quartz window of the cell containing the colloid sol under investigation.

The cell which is used is illustrated in Fig. 30, the beam of light

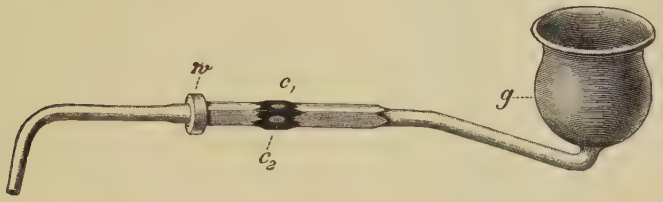


FIG. 30.—Cell with quartz windows for the Siedentopf-Zsigmondy slit ultramicroscope.

passing through quartz window, (c_2), the microscope through which the reflected beam passes to the observer's eye being focused through the quartz window, (c_1). Figure 31 shows diagrammatically the appearance of the beam of light passing through window (c_2), into the colloid sol. The micrometer screws, (*l*) and (*m*), Fig. 27, adjust the focus of the

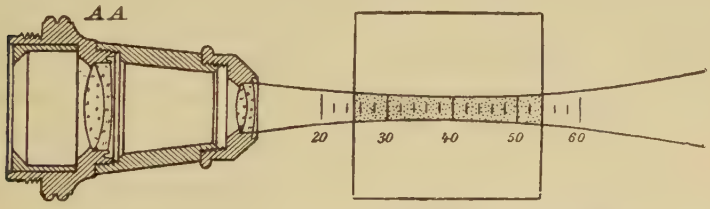


FIG. 31.—The appearance of the illuminated area of colloid particles in the cell of the Siedentopf-Zsigmondy slit ultramicroscope.

objective, (*AA*), Fig. 31, until the beam of light comes to a focus in the center of the visible field, as shown in Fig. 31.

Burton[91] has provided an excellent discussion of the precautions that must be observed in order to make studies with the ultramicroscope. Similarly, the precautions noted by Zsigmondy[92] should be taken care-

[91] Burton, E. F., The Physical Properties of Colloidal Solutions, Second Edition, Chap. III, Longmans, Green and Company, New York (1921).

[92] Zsigmondy, R., Colloids and the Ultramicroscope, translated by Jerome Alexander, John Wiley & Sons, Inc. (1909).

fully into consideration, providing the instrument is to be used as a research tool. The photomicrograph reproduced in Fig. 19 was secured by making use of an ultramicroscope.

Other forms of the ultramicroscope which are extremely useful in biological investigations are those known as the paraboloid condenser or the still more improved type, the cardioid condenser, as developed by Siedentopf.[93,94] Both the paraboloid condenser and the cardioid condenser are designed so as to fit directly in the frame of the ordinary high-grade microscope replacing the ordinary sub-stage condenser. Figure 32 shows diagrammatically the path of the light rays through a paraboloid condenser. The shaded area, (*d*), is an optical glass block, the curved faces of which are highly silvered so as to form reflecting mirrors. A black glass disc is inserted over a portion of the lower surface so as to provide a black background directly under the objective of the microscope. The light rays, (*A*) and (*A'*), reflected from the mirror of the microscope, pass through the clear portion of the optical glass block and are reflected from the mirror surfaces at such an angle that they focus at a point slightly below the plane of contact of the glass slide, (*E*), with the cover glass, (*F*). The slide, (*E*), contains a slight depression in which the colloidal system under investigation is placed, the light rays coming to focus within the colloid system lying in this depression. The microscope objective is then focused on the point at which the light rays converge within this depression.

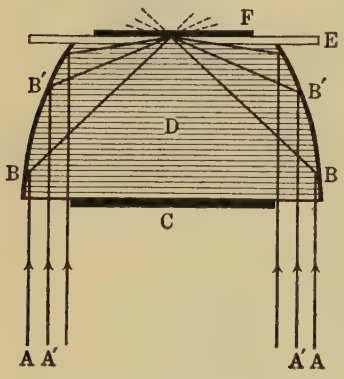


FIG. 32.—A diagrammatic representation of the path of the light rays in a paraboloid condenser.

Figure 33 shows the more elaborate and more nearly optically perfect arrangement of lenses in the cardioid condenser. The light rays in this instance are focusing in a block of uranium glass which shows strong fluorescence. One precaution which must always be observed when either the paraboloid condenser or the cardioid condenser is used is to make contact of the microscope slide with the surface of the condenser through the medium of an immersion oil. If the oil is not used, the rays

[93] Siedentopf, H., Über ultramikroskopische Abbildung, *Z. f. wiss. Mikr.*, 26 : 391–410 (1909), und Über einen neuen Fortschritt in der Ultramikroskopie, *Ber. deut. physik. Ges.*, 8 : 6–47 (1910).

[94] Siedentopf, H., Über bisphärische Spiegelkondensoren für Ultramikroskopie, *Ann. d. Physik* (4) 39 : 1175–1184 (1912).

will not pass from the surface of the condenser into and through the glass slide, due to refraction at a glass-air interface.

The limit of the size of the particle which becomes discernible in the ultramicroscope depends upon two factors, the intensity of illumination and the difference in refractive index existing between the disperse phase and the dispersions medium.

Using direct sunlight and extreme precautions, Zsigmondy was able to differentiate particles of colloidal gold as small as $1.7m\mu$ from the water in which they were dispersed. As Siedentopf[95] points out, if molecules could be gotten far enough apart and if strong enough illumination were possible, molecules themselves could be differentiated in the ultramicroscope. He notes, however, that sunlight is far too dim a light source, and since sunlight is the most intense source of light which we have available, the theoretical possibilities of the ultramicroscope cannot be attained. The usual lower limit of resolution of the Siedentopf-Zsigmondy ultramicroscope lies at about $5m\mu$ under ordinary conditions of illumination, with a somewhat higher value for the cardioid form of the ultramicroscope and a still somewhat higher value for the paraboloid condenser.

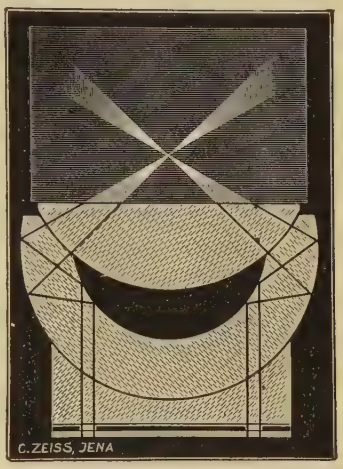


FIG. 33.—The path of the light rays in a cardioid condenser. A uranium glass plate has been placed above the condenser to show the focus of the rays which have passed through the condenser. Where these rays converge is the point upon which the microscope should be focused for the study of colloid systems.

As noted above, the slit ultramicroscope can be utilized to determine approximately the size of the colloid particle on the assumption that the particles in question are spheres and that the specific gravity of the colloidal micelle is identical with the specific gravity of the same material in mass. Using the rotating slit, a definite volume of a dilute sol can be illuminated, and the number of points of light, each indicating the position of a particle in this volume, can be counted. The weight of the disperse phase in the original sol can be ascertained by evaporating a portion of the sol to dryness and determining its solid content.

Let us assume, for example, that a silver sol was prepared which contained 6.8 mg. of silver in 100 cc. volume. The original sol was diluted

[95] Siedentopf, H., On the Rendering Visible of Ultra-Microscopic Particles and of Ultra-Microscopic Bacteria, *J. Roy. Microscopical Soc.*, 573–582 (1903).

100 times, and when viewed in the ultramicroscope was found to contain 300 particles per 0.1 cu. mm. The original sol, therefore, contained 30,000,000,000 particles, weighing a total of 6.8 mg. The mean volume of the particles in the sol was 2.2×10^{-14}cc., assuming a specific gravity of 10.5. If the particles are spheres with an average radius, r, we have

$$\tfrac{3}{4}\pi r^3 = 22 \times 10^{-15}. \qquad (23)$$

Accordingly $r = 2.1 \times 10^{-5}$cm. or 0.21μ. The silver particles, therefore, lie at the extreme upper limit of the colloid realm insofar as their size is concerned.

The errors in such a measurement are (1) the difficulty of accurately counting the number of particles, due to their incessant Brownian movement, (2) the fact that the particles may not be of uniform size (some may be microns, some ultramicrons, some amicrons, and a part of the material in the disperse phase may be even molecularly dispersed), and (3) the fact that the size of the particle may be affected by dilution.

A second method depends upon a count of the number of particles in a definite volume, comparing this with the total mass of material in a given amount of the sol, and a measurement of the distance between the particles as viewed in the ultramicroscope. The formula which is utilized here is

$$r = \sqrt[3]{\frac{M}{d}}x \qquad (24)$$

where M = the weight of disperse phase in a unit volume of the sol;
d = density of the material making up the disperse phase;
x = the average distance in centimeters between the particles comprising the disperse phase.

The same errors apply to this method as to the method above.

Relatively few studies have been conducted on biological material, making use of ultramicroscopic technic. If the protoplasm of a *Spyrogira* cell is viewed in an ultramicroscope, it is found to be full of light points in regions which are optically void when viewed by ordinary microscopic technic. Undoubtedly the ultramicroscope, particularly in the form of the paraboloid condenser, affords a tool which the biologist cannot afford to ignore.

CHAPTER IV

HYDROGEN ION CONCENTRATION

In order to discuss adequately certain properties of colloid systems and to understand certain biological reactions, it is necessary to diverge at this point and consider briefly the subject of hydrogen ion concentration.

It is impossible within the space at our disposal to cover adequately all of the details regarding hydrogen ion concentration, including the theoretical background and all of the technic which is involved in the various methods used for measurement. We are particularly fortunate in having available such excellent treatises as those of Clark[1] and Michaelis,[2,3] which are indispensable adjuncts to the library of anyone working in this field.

Water is in many respects a unique substance. Thus, for example, it is liquid at ordinary temperatures, whereas compounds more or less similar in structure, such as H_2S, SO_2, NH_3, etc., are gaseous; in the liquid state it has a very high surface tension differentiating it from other liquids; it has a minimum volume at $+4^\circ$C., expanding on solidifying; and last but not least, solutions of many substances in water as a solvent possess the ability to conduct an electric current. If acids or bases or salts are dissolved in water, the solution becomes a conductor for an electric current. Accordingly, such substances are known as electrolytes.

Arrhenius, in 1887, postulated that when electrolytes were dissolved in water they were dissociated into their corresponding ions and that it was these charged ions through which the flow of electric current took place. The separation of an electrolyte into its component ions is known as *dissociation.*

Arrhenius noted that electrolytic conductance in solution was not strictly proportional to the amount of electrolyte which was dissolved.

[1] Clark, W. Mansfield, The Determination of Hydrogen Ions, Third Edition, Williams and Wilkins Company, Baltimore (1928).

[2] Michaelis, L., Hydrogen Ion Concentration, Vol. I. Principles of the Theory, translated by W. A. Perlzweig, Williams and Wilkins Company, Baltimore (1926).

[3] Michaelis, L., Die Wasserstoffionenkonzentration, Julius Springer, Berlin (1914).

He accordingly suggested that at infinite dilution complete dissociation took place, whereas in more concentrated solutions the dissociation was not entirely complete, part of the original solute remaining in solution in an undissociated condition. In accordance with this view, if we dissolve sodium chloride in water, the following equilibrium would be set up:

$$NaCl \rightleftharpoons Na^+ \text{ and } Cl^-.$$

In a dilute solution the equilibrium would be shifted toward the right until at infinite dilution all of the sodium chloride was dissociated. In a concentrated solution the equilibrium would be shifted more and more toward the left.

The views of Arrhenius in regard to the dissociation of electrolytes have been altered somewhat by the recent researches of physicists and physical chemists, which have thrown doubt on the existence of individual molecules, such as is represented by NaCl. The X-ray crystal structure of sodium chloride does not indicate the presence of a definite molecule of NaCl, but rather of atoms of sodium and chlorine spaced at equal distances from each other throughout the crystal structure. Accordingly, it has been suggested by some that an electrolyte such as sodium chloride is completely dissociated even in the solid state. If this be the case, it must be obvious that it would also be completely dissociated in water. It would, therefore, be theoretically impossible to have a solution where the sodium chloride was 98 per cent dissociated. Solutions of sodium chloride can, however, be obtained which have only 98 per cent of the electrolytic conductance which is theoretically possible. Accordingly a new term has recently come into chemical literature, *i.e.*, the term, *activity*, which is rather generally displacing the term, dissociation, when solutions of strong electrolytes are under consideration. On the assumption that dilute solutions of strong electrolytes are completely dissociated but that as the concentration increases, the anions are more or less decreased in their activity by the adjacent cations, and correspondingly the activity of the cations may be altered by the adjacent anions, a theory has been built up substituting changes in activity for the older conception of changes in dissociation. Whether or not this new theory will stand the test of time remains to be proven. Undoubtedly it does not tell the whole story, inasmuch as even its most enthusiastic sponsors limit the applicability of the theory to the strong electrolytes and still retain the theory of incomplete dissociation to explain the behavior of solutions of weak electrolytes, such as acetic acid or ammonia.

The above discussion is inserted at this point merely to indicate some of the recent trends. It is probably more strictly correct to speak of

hydrogen ion activity than it is to speak of *hydrogen ion concentration*, and throughout the following discussion it will be well to bear in mind that the term hydrogen ion concentration refers to the "apparent" hydrogen ion concentration, *i.e.*, the hydrogen ion activity, rather than to the actual normality of ionized hydrogen present in the solution, for the methods which are employed for the measurement of hydrogen ion concentration in reality measure the so-called hydrogen ion activity.[4]

If we have an acid of the type HA, its dissociation into H^+ and A^- is reversible and the dissociation may be expressed as

$$HA \rightleftharpoons H^+ \text{ and } A^-$$

Applying the equation for mass action and expressing concentration by inclosing the quantities in brackets, we have the equation,

$$\frac{[H^+] \times [A^-]}{[HA]} = K_a \tag{25}$$

where K_a = the so-called ionization or dissociation constant.

Equation (25) states that for any given acid the product of the concentration of the anion and the cation divided by the concentration of the remaining undissociated acid is a constant. It will be obvious from the above equation that if K_a is large, the greater part of the acid is dissociated into hydrogen ions and anions, whereas if K_a is small, the greater part of the acid is present in the form of undissociated molecules. Accordingly the dissociation constant K_a is a measure of the relative strength of an acid.

In a similar way the dissociation of a base may be represented by

$$\frac{[B^+] \times [OH^-]}{[BOH]} = K_b \tag{26}$$

where K_b = the dissociation constant of a base and bears the same relation in regard to the alkalinity of a solution that K_a has with respect to the acidity.

Inasmuch as water is the common solvent and inasmuch as it dissociates somewhat into hydrogen and hydroxyl ions, one of which is

[4] For those who wish to go further into the theories underlying the activity of ions, the following books are recommended: Eucken, A., Jette, Eric R., and LaMer, V. K., Fundamentals of Physical Chemistry, McGraw–Hill Book Company, New York (1925), and Lewis, G. N., and Randall, M., Thermodynamics and the Free Energy of Chemical Substances, McGraw–Hill Book Company, New York (1923). See also Lewis, G. N., and Randall, M., The Activity Coefficient of Strong Electrolytes, *J. Am. Chem. Soc.*, 43 : 1112–1154 (1921), and The Theory of Strong Electrolytes, Faraday Society, London (1927).

characteristic of the dissociation of acids, the other of bases, the dissociation constant of water becomes a very important quantity in the calculation of hydrogen ion concentration. The dissociation of water may be represented by

$$\frac{[H^+] \times [OH^-]}{[HOH]} = K_w \tag{27}$$

This equation states that the product of the concentration of hydrogen and hydroxyl ions divided by the concentration of the undissociated water is a constant. It is usually regarded as safe to ignore the denominator in this equation, inasmuch as it appears to be approximately constant. The equation accordingly is usually expressed as

$$[H^+] \times [OH^-] = K_w \tag{28}$$

where K_w = the dissociation constant of water.

Gortner, Hoffman, and Sinclair[5, 6] have recently raised the question as to whether the actual concentration of H_2O can be ignored in the calculation of the dissociation constant of water, pointing out that if water is a mixture of a number of molecular species, it may be possible that only mono-hydrol (H_2O) undergoes dissociation into hydrogen and hydroxyl ions

$$(H_2O)_n \rightleftharpoons (H_2O)_3 \rightleftharpoons (H_2O)_2 \rightleftharpoons H_2O \rightleftharpoons H^+OH^-.$$

If only a small amount of mono-hydrol were present, there might be an appreciable change in its concentration due to dissociation, in which case the concentration of H_2O could not be regarded as a negligible factor in the calculation of K_w. Since, however, there is no known means of determining the relative proportions of water molecules in their different degrees of polymerization, it is impossible at the present time to test the above hypothesis.

From equation (28) we read that no matter how great is the concentration of hydrogen ions, there must always remain sufficient hydroxyl ions in the solution to satisfy the equation, and conversely no matter how great the concentration of hydroxyl ions, there must always be a residue of hydrogen ions present to satisfy the above equation. The value for the dissociation constant of water is, therefore, a very important constant, and its value is approximately 10^{-14}. It is usually

[5] Gortner, R. A., Hoffman, W. F., and Sinclair, W. B., Physico-chemical Studies on Proteins. III. Proteins and the Lyotropic Series, Colloid Symposium Monograph, Vol. V, pp. 179–198, Chemical Catalog Company, Inc., New York (1928).

[6] Gortner, R. A., Hoffman, W. F., and Sinclair, W. B., Zur Kenntnis der Proteine und der lyotropen Reihen, *Koll. Z.*, 44 : 97–108 (1928).

expressed as $\log \frac{1}{K_w}$ which has a value of 13.995 at 22° C.[7] If K_w at 22° C. has a value of 1×10^{-14} and the hydrogen and hydroxyl ion concentrations are equal, then pure water at this temperature is 1×10^{-7} $\left(\text{or } \frac{1}{10{,}000{,}000}\right)$ normal with respect to hydrogen and hydroxyl ions. One gram molecule of any substance contains approximately 6.061×10^{23} molecules. Accordingly in pure water there would be $6.061 \times 10^{23} \times 10^{-7}$ hydrogen ions per liter. One liter of water contains 55.56 gram molecules or $55.56 \times 6.061 \times 10^{23}$ water molecules. This same liter, on the other hand, contains only 6.061×10^{16} hydrogen ions. Accordingly only one molecule of water in every 555,000,000 molecules is dissociated into hydrogen and hydroxyl ions.

From equations (25), (26), and (28) it is possible to present an equation which applies equally well to all solutions of acids and bases. Equation (25) may be rewritten

$$\frac{1}{[H^+]} = \frac{[A^-]}{K_a[HA]} \tag{29}$$

or it may be expressed by using the reciprocal of $[H^+]$ and taking the logarithm of each side of the equation as

$$\log \frac{1}{[H^+]} = \log \frac{1}{K_a} + \log \frac{[H^-]}{[HA]} \tag{30}$$

The logarithm of the reciprocal of the hydrogen ion concentration is expressed in the term pH $\left(p\text{H} = \log \frac{1}{[H^+]}\right)$. The term pH, which is commonly met with in biological and biochemical studies where hydrogen ion concentration is referred to, was first introduced by Sörensen[8,9] for reasons which will be referred to later.

By referring to equation (28) it will be noted that the term pH can

[7] Clark, *loc. cit.*, gives on page 45 a table for the variation of $\log \frac{1}{K_w}$ with temperature, together with the pH of the neutral point at the various temperatures.

[8] Sörensen, S. P. L., Enzymstudien. II. Über die Messung und die Bedeutung der Wasserstoffionen-konzentration bei enzymatischen Prozessen, *Biochem. Z.*, 21 : 131–304 (1909).

[9] Sörensen, S. P. L., Études enzymatiques. II. Sur la mesure et l'importance de la concentration des ions hydrogène dans les réactions enzymatiques, *Compt. rend. Lab. Carlsberg*, 8 : 1–168 (1909).

be used equally well to express either the degree of acidity or the degree of alkalinity of a solution, inasmuch as that equation could be rewritten

$$\frac{1}{[H^+]} \times \frac{1}{[OH^-]} = \frac{1}{K_w} \tag{31}$$

or

$$p\mathrm{H} + p\mathrm{OH} = p\mathrm{K}_w \tag{32}$$

When a substance yielding H^+ or OH^- ions is added to water, the ionization of the water is repressed so that the hydrogen ion concentration decreases as the hydroxyl ion concentration increases and vice versa. Therefore *a decrease in hydrogen ion concentration may be used to express an increase in hydroxyl ion concentration, thus permitting one scale to be used for the measurement of both acidity and alkalinity.*

The fact that the symbol *p*H can be used to designate either acidity or alkalinity makes it especially useful in biochemical studies where the degree of acidity or alkalinity ranges around the neutral point. Table VIII shows the relationship between *p*H and the concentration of hydrogen or hydroxyl ions which may be present in solution.

An additional reason for using the symbol *p*H lies in the fact that we can express in 14 units, acidities which range from a solution which is 0.1 normal in terms of hydrogen ions to a solution which is one one-hundred trillionth normal, (10^{-14}), in terms of hydrogen ions. If we desired to plot the actual hydrogen ion concentration, C_H, or the hydrogen ion activity, C_{Ha}, allowing 1 mm. for the unit between 10^{-13} normal and 10^{-14} normal, we would need a piece of paper 111,111 + kilometers or approximately 69,444 miles long in order to include the whole range of C_H

TABLE VIII

Showing the Relationship between *p*H, *p*OH, and the Normality of a Solution in Terms of Active Hydrogen or Hydroxyl Ions

*p*H 1	= 0.1 N	Hydrogen Ions and 10^{-13} N	Hydroxyl Ions = *p*OH 13
*p*H 2	= 0.01 N	Hydrogen Ions and 10^{-12} N	Hydroxyl Ions = *p*OH 12
*p*H 3	= 0.001 N	Hydrogen Ions and 10^{-11} N	Hydroxyl Ions = *p*OH 11
*p*H 4	= 0.0001 N	Hydrogen Ions and 10^{-10} N	Hydroxyl Ions = *p*OH 10
*p*H 5	= 10^{-5} N	Hydrogen Ions and 10^{-9} N	Hydroxyl Ions = *p*OH 9
*p*H 6	= 10^{-6} N	Hydrogen Ions and 10^{-8} N	Hydroxyl Ions = *p*OH 8
*p*H 7	= 10^{-7} N	Hydrogen Ions and 10^{-7} N	Hydroxyl Ions = *p*OH 7
*p*H 8	= 10^{-8} N	Hydrogen Ions and 10^{-6} N	Hydroxyl Ions = *p*OH 6
*p*H 9	= 10^{-9} N	Hydrogen Ions and 10^{-5} N	Hydroxyl Ions = *p*OH 5
*p*H 10	= 10^{-10} N	Hydrogen Ions and 0.0001 N	Hydroxyl Ions = *p*OH 4
*p*H 11	= 10^{-11} N	Hydrogen Ions and 0.001 N	Hydroxyl Ions = *p*OH 3
*p*H 12	= 10^{-12} N	Hydrogen Ions and 0.01 N	Hydroxyl Ions = *p*OH 2
*p*H 13	= 10^{-13} N	Hydrogen Ions and 0.1 N	Hydroxyl Ions = *p*OH 1
*p*H 14	= 10^{-14} N	Hydrogen Ions and 1.0 N	Hydroxyl Ions = *p*OH 0

between *p*H 1 and *p*H 14. In terms of *p*H the entire graph could be placed on an ordinary sheet of coordinate paper. *It is essential to remember that the pH scale is a logarithmic scale.* Accordingly *p*H 6 represents a solution containing 10 times the concentration of hydrogen ions that are present in a solution having a *p*H of 7. A solution with a *p*H of 5 is 100 times as acid as a solution with a *p*H of 7. A solution with a *p*H of 4 is 1000 times as acid as a solution with a *p*H of 7. Likewise *the fractions in a pH scale do not represent arithmetical values but rather logarithmic values.* Thus, the difference in acidity between *p*H 5.0 and 5.1 is many times greater than is the difference between *p*H 5.9 and *p*H 6.0. A rather graphic illustration of the relationship between degree of acidity at various points on the *p*H scale is shown in Table IX, where the dimensions of cubical boxes which would vary in the same ratio as the hydrogen ion concentration are compared with *p*H values.

TABLE IX

Hypothetical Comparison of the Various Points of the *p*H Scale with Cubical Containers Varying in Size in Similar Numerical Ratios

	Edge of Cube, Inches		Edge of Cube, Inches
*p*H 6 to *p*H 7.....	$\frac{1}{4}$	*p*H 2 to *p*H 3.....	$5\frac{5}{16}$
*p*H 5 to *p*H 6.....	$\frac{1}{2}\frac{7}{2}$	*p*H 1 to *p*H 2.....	$11\frac{9}{32}$
*p*H 4 to *p*H 5.....	$1\frac{5}{32}$	*p*H 0 to *p*H 1.....	25
*p*H 3 to *p*H 4.....	$2\frac{1}{2}$		

Expressing acidity or alkalinity in terms of the concentration of the hydrogen or hydroxyl ions or in terms of *p*H permits us to distinguish between the strong acids or bases and the so-called weak acids or bases. Hydrochloric acid and acetic acid dissociate respectively as follows:

$$HCl \rightleftharpoons H^+ \text{ and } Cl^-$$

$$HC_2H_3O_2 \rightleftharpoons H^+ \text{ and } C_2H_3O_2^-.$$

Hydrochloric acid, however, is a strong acid and ion concentration measurements indicate that all or practically all of the hydrogen ions in an 0.1 normal solution of hydrochloric acid are active. On the other hand, only a small fraction of the available hydrogen ions in acetic acid are active (less than 1 per cent in an 0.1 normal solution). We accordingly say that only a small fraction of the acetic acid is dissociated, the major portion remaining as undissociated acetic acid molecules.

If we were to titrate deci-normal solutions of acetic and hydrochloric

acids with standard sodium hydroxide, using a suitable indicator, we would find that both acids required the same amount of sodium hydroxide for neutralization. The amount of standard sodium hydroxide which would be required represents the *titratable acidity*, but this determination tells us nothing in regard to the *actual acidity* of the solution at any particular period of time. *The titratable acidity is a measure of the potential acidity, i.e., the quantity of hydrogen ions which can be made to combine with a base. Hydrogen ion concentration measurement, on the other hand, is a measure of the actual concentration (or activity) of hydrogen ions present in a given system at a given time, and is therefore the only true measure of how such a solution will affect another system which is sensitive to hydrogen or hydroxyl ions.* For example, hydrochloric acid is a strong acid and its hydrogen ions in solution approach ideal activity. A normal solution of hydrochloric acid is a poison, whereas a normal solution of acetic acid is a fairly weak artificial vinegar. Both solutions have the same potential (*i.e.*titratable) acidity but their hydrogen ion concentrations are widely different.

The reactions of the biological organism toward acids and bases are not controlled by changes in potential acidity but rather are affected by changes in the actual concentration of hydrogen ions. Table X, taken from the

TABLE X

The Approximate Relationship between the Normality of Acid Solutions (Potential Acidity) and the *p*H (the Reciprocal of the Actual Acidity) at 25° C.*

Normality of Solution	*p*H of Acid Solution at 25° C.				
	Lactic	Acetic	Ortho-Phosphoric	Hydrochloric	Oxalic
N/2	1.89	2.50	1.33	0.46	1.03
N/5	2.11	2.69	1.59	0.81	1.29
N/10	2.27	2.85	1.79	1.09	1.52
N/25	2.49	3.08	2.05	1.46	1.82
N/50	2.67	3.24	2.26	1.77	2.10
N/100	2.82	3.39	2.49	2.06	2.36
N/200	3.00	3.54	2.73	2.35	2.62
N/500	3.23	3.76	3.08	2.73	3.00

* The values for *p*H are probably not exact from the standpoint that they could be used as physico-chemical constants. They represent, however, actual values which were obtained on the solutions in question by using electrometric methods of determining hydrogen ion concentration. These values should not be used except as representing first approximations and are inserted here largely for the purpose of illustrating the variation which may be obtained in actual acidity when solutions of the same potential acidity are compared.

data of Sharp and Gortner,[10] shows the relationship between the potential acidity and the actual acidity of solutions of certain of the common acids.

Figures 34 and 35, taken from the data of Gortner and Sharp[11] illustrate in a graphic way the differences which may be observed in biochemical systems which are sensitive to the action of acids. In Fig. 34 the viscosity of a 20 per cent wheat flour-in-water suspension is plotted against the normality of the various acids which were added to

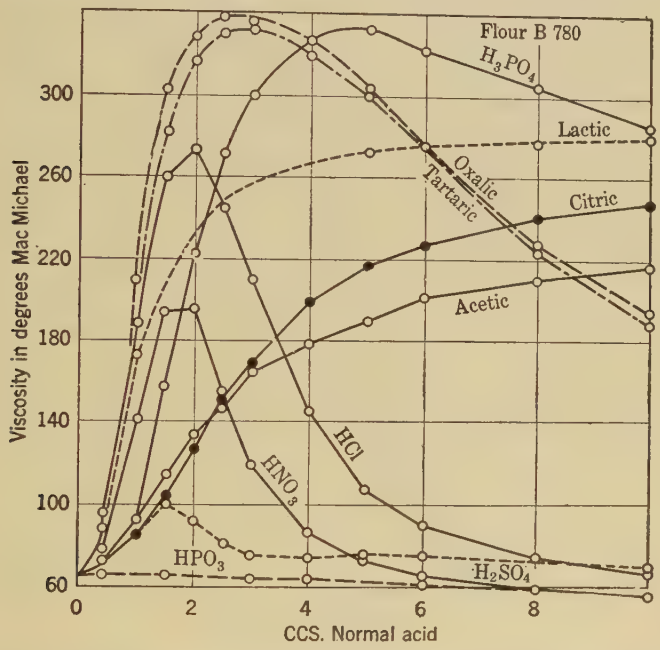


FIG. 34.—Showing the viscosity of flour-in-water systems plotted against the normality of solutions of the various acids. (Data of Gortner and Sharp.)

the suspension. It will be noted that there is a great difference in the form of the viscosity curves with acid concentration. When, however, the above data were plotted not against the potential acidity of the solution but against the actual acidity, *i.e.*, the hydrogen ion concentra-

[10] Sharp, P. F., and Gortner, R. A., Physico-chemical Studies of Strong and Weak Flours. II. The Imbibitional Properties of the Glutens from Strong and Weak Flours, *J. Phys. Chem.*, 26 : 101–136 (1922).

[11] Gortner, R. A., and Sharp, P. F., The Physico-chemical Properties of Strong and Weak Flours. III. Viscosity as a Measure of Hydration Capacity and the Relation of the Hydrogen Ion Concentration to Imbibition in the Different Acids, *J. Phys. Chem.*, 27 : 481–492 (1923).

tion, Fig. 35 resulted. It will be noted that instead of a maximum being reached at various points on the curve, as in Fig. 34, the maximum viscosity is in all instances reached at an acidity of approximately *p*H 3.0, and the acids instead of behaving in entirely dissimilar manners yield curves of essentially the same general shape. It is obvious from an inspection of these two figures that the normality of the acid which was added was not the true variable affecting the system, but that the hydrogen ion concentration was in reality the variable concerned.

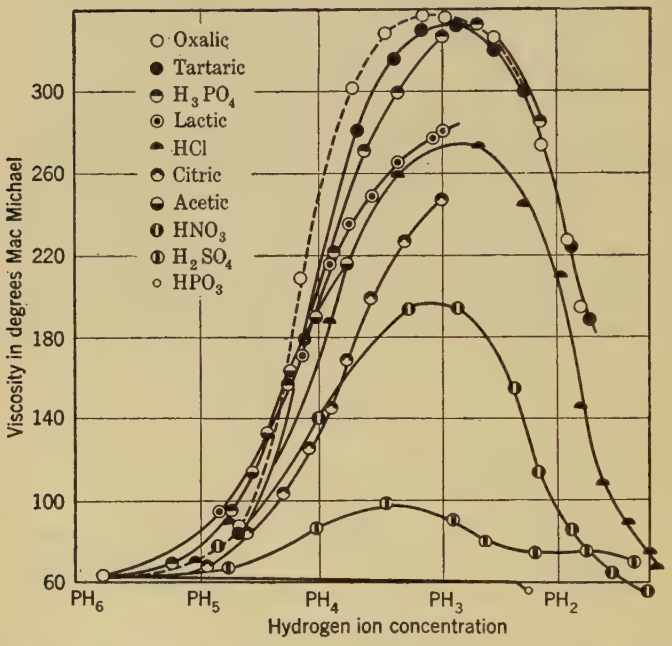


Fig. 35.—Showing the same data as in Fig. 34, plotted in terms of actual hydrogen ion concentration.

It is beyond the scope of this book to more than mention the methods by which hydrogen ion concentration can be measured. As noted at the beginning of this chapter, the excellent manuals of Clark and Michaelis are adequate in all respects. The two methods generally employed are colorimetric measurements and electrometric measurements.

Colorimetric Measurements.—In the measurement of hydrogen ion concentration by colorimetric methods one is concerned with the change in color of an indicator as a measurement of the change in hydrogen ion concentration. Indicators are in general compounds

TABLE XI

Indicators Recommended for the Colorimetric Determination of Hydrogen Ion Concentration Together with Their Usable *p*H Range and Certain Other Physico-Chemical Constants *

Chemical Name	Common Name	Molecular Weight	*A*	*pK*	Range	Color Change		*B*	*C*	Absorption Max.	
						Acid	Alkaline			Acid	Alkaline
					*p*H			*p*H	*p*H	$m\mu$	$m\mu$
3.3′ dimethyl phenolsulfonphthalein	Meta cresol purple	382	26.2	1.51	1.2–2.8	Red	Yellow	conc. HCl	6	533	
2.2′ isopropyl 5.5′ dimethyl phenolsulfonphthalein	Thymol blue	466	21.5	1.5	1.2–2.8	Red	Yellow	conc. HCl	6	544	
2.2′ 6.6′ tetra brom phenolsulfonphthalein	Brom phenol blue	669	14.9	3.98	3.0–4.6	Yellow	Blue	0	7	.....	592
2.2′ 6.6′ tetra brom 3.3′ dimethyl phenolsulfonphthalein	Brom cresol green	698	14.3	4.67	3.8–5.4	Yellow	Blue	1	8	.....	617
2.2′ dichlor phenolsulfonphthalein	Chlor phenol red	423	23.6	5.98	4.8–6.4	Yellow	Red	2	9	.....	573
Ortho carboxy benzene azo dimethyl aniline	Methyl red	279	35.6	.....	4.4–6.0	Red	Yellow				
2.2′ dibrom phenolsulfonphthalein	Brom phenol red	512	19.5	6.16	5.2–6.8	Yellow	Red	3	10	.....	574
5.6′ dibrom 2.2′ dimethyl phenolsulfonphthalein	Brom cresol purple	540	18.5	6.3	5.2–6.8	Yellow	Purple	3	10	.....	591
6.6′ dibrom 2.2′ isopropyl 5.5′ methyl phenolsulfonphthalein	Brom thymol blue	624	16.0	7.0	6.0–7.6	Yellow	Blue	4	10	.....	617
Phenolsulfonphthalein	Phenol red	354	28.2	7.9	6.8–8.4	Yellow	Red	5	11	.....	558
2.2′ dimethyl phenolsulfonphthalein	Cresol red	382	26.2	8.3	7.2–8.8	Yellow	Red	5	11	.....	572
3.3′ dimethyl phenolsulfonphthalein	Meta cresol purple	382	26.2	8.32	7.4–9.0	Yellow	Purple	5	11	.....	580
2.2′ isopropyl 5.5′ dimethyl phenolsulfonphthalein	Thymol blue	466	21.5	8.9	8.0–9.6	Yellow	Blue	6	12	.....	596
Ortho cresol phthalein	Cresol phthalein	.....	.....	(9.4)	8.2–9.8	Colorless	Red	6	12		

A = cubic centimeters of 0.01 N NaOH required per 0.1 gram indicator to form mono sodium salt. Dilute to 250 cc. for 0.04 per cent reagent.
B = approximate *p*H value of solution required for full "acid color" appertaining to *p*H range indicated.
C = approximate *p*H value of solution required for full "alkaline color" appertaining to *p*H range indicated.

* From W. M. Clark, "The Determination of Hydrogen Ions, Third Edition," Williams and Wilkins Company, Baltimore. 1928. By permission.

which form salts with either acids or bases and yield on dissociation at least one colored ion. Accordingly the degree of dissociation of the indicator is altered by changes in hydrogen or hydroxyl ion concentration. The concentration of the colored ion is accordingly altered, thus causing a change in the depth of color of the indicator as measured in a colorimeter. Various indicators have been suggested for the various *p*H ranges. Table XI shows the indicators which are most suitable, together with their color changes from acid to base and the *p*H range in which they can be used.

The phenolsulfonphthalein group of indicators are triphenyl methane derivatives containing a sulfophenyl and two phenol radicals, the latter being attached in their para positions to the methane carbon,

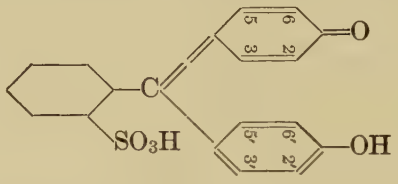


Figure 36[12] shows the dissociation curve of the indicators noted in Table XI, the heavily shaded portion of the curve representing the range over which the indicator is useful. It will be noted that the indicator is in general most useful when it is approximately 50 per cent dissociated.

Electrometric Measurements.—Electrometric determinations are based on the assumption that ions in solution obey the gas laws. If we allow a metal electrode to dip into a solution containing ions of that metal, the partial pressure of the ions in solution will be proportional to their ionic concentration. If the electrode be of such size that one gram molecule of ions carrying nF faraday of electricity passes from the electrode to the solution, the partial pressure of the solution is raised by the amount dP, and a difference in potential is observed between the electrode and the solution which is equal to dE. The electrical energy which is expended will then be $nFdE$, and the work done upon the system will be VdP. Assuming that the process is reversible, the system comes to equilibrium where the amount of energy expended is equal to the amount of work done. Accordingly,

$$nFdE - VdP = 0 \qquad (33)$$

[12] From Clark, W. Mansfield, The Determination of Hydrogen Ions, Third Edition, Williams and Wilkins Company, Baltimore (1928). Reprinted by permission.

and from the gas laws $VP = RT$, or $V = \frac{RT}{P}$, we can write,

$$dE = \frac{RT}{nF} \cdot \frac{dP}{P} \tag{34}$$

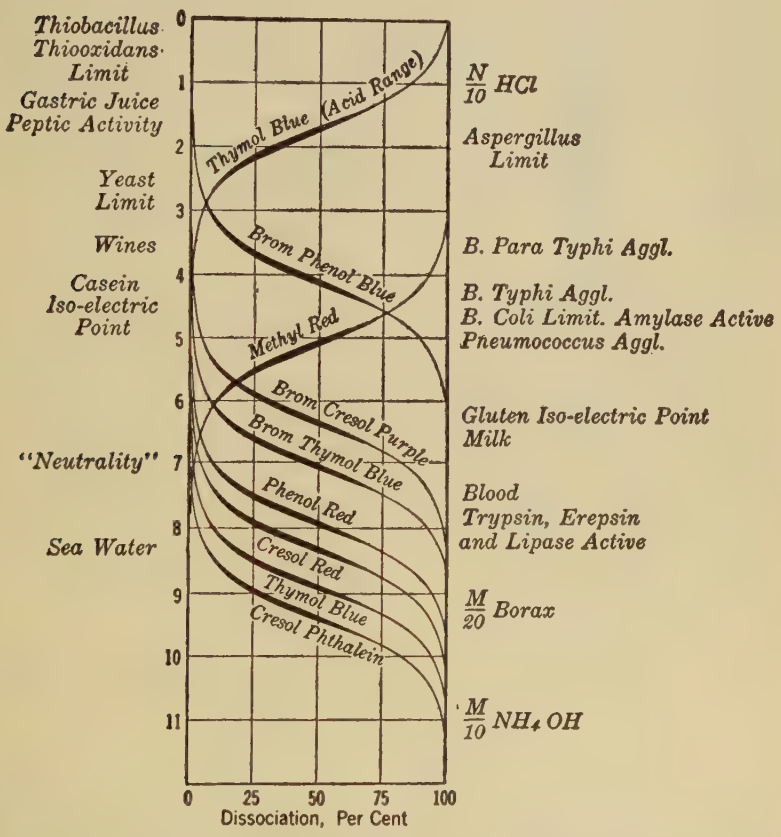


FIG. 36.—The dissociation curves for the most useful indicators for the colorimetric estimation of hydrogen ion concentration. The shaded areas indicate the useful range. (From Clark's "Determination of Hydrogen Ions." By permission.)

This by integration becomes,

$$E = \frac{RT}{nF} \log_e P + K \tag{35}$$

where K = an integration constant.

The integration constant is the difference in potential between the electrode and the solution when the pressure differs by some

arbitrary unit. The Nernst equation, expressing the above, can be written

$$E = \frac{RT}{nF} \log_e \frac{P}{p} \tag{36}$$

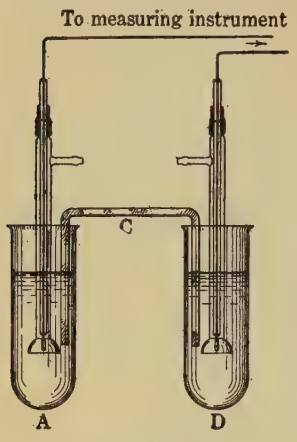


FIG. 37. — A possible "gas chain" arrangement in which A is the standard solution, D the test solution, and C a connection tube filled with an agar gel in a neutral, conducting solution (*e.g.*, KCl solution). The hydrogen electrode potential in A remains fixed, while that in D varies with the acidity of the solution in D. (After Klopsteg.)

If we have two vessels arranged as in Fig. 37, the liquids in the two vessels differing in concentration, and these are connected in the manner shown in the figure, there will be set up a difference in potential between the two electrodes which can be detected by use of a suitable measuring device. Electrode (1) will have a potential $E = \frac{RT}{nF} \log_e P + K$, and electrode (2) a potential of $E' = \frac{RT}{nF} \log_e P' + \mathrm{K}$, the potentials accordingly varying in direct relation to the osmotic pressure or to the concentration of ions in the solution. If P' is less than P, the electrode E' will be negative to the electrode E. The electromotive force which is set up can be expressed as

$$\text{E.M.F.} = E - E' \tag{37}$$

or

$$\text{E.M.F.} = \frac{RT}{nF} \log_e P + K - \frac{RT}{nF} \log_e P' + K \tag{38}$$

or

$$\text{E.M.F.} = \frac{RT}{nF} \log_e \frac{P}{P'} \tag{39}$$

and since the osmotic pressures are proportional to the ion concentrations, it may be further written as

$$\text{E.M.F.} = \frac{RT}{nF} \log_e \frac{C}{C'} \tag{40}$$

where C and C' represent the concentrations of the ions in the two solutions in question.

R = the gas constant;
T = the absolute temperature;
n = the valency of the ion;
F = the faraday or 96,500 coulombs.

The hydrogen electrode is nothing more than a metallic electrode which has been coated with a thin layer of platinum black deposited on an inactive metal and exposed to an atmosphere of hydrogen sufficiently long for the platinum black to become completely saturated with hydrogen. When such an electrode is placed in a solution containing hydrogen ions, it reaches an equilibrium with the solution which varies with the concentration of the hydrogen ions in the solution, and behaves as if it were an electrode composed of metallic hydrogen. Figure 38 shows

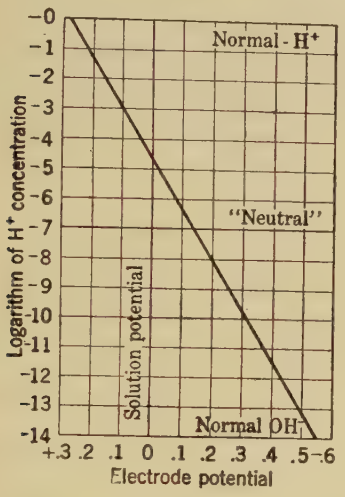


Fig. 38.

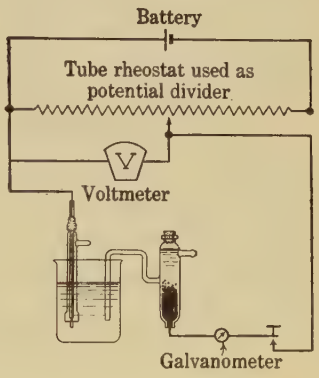


Fig. 39.

Fig. 38.—The electrode potential of the hydrogen electrode with reference to the solution in which it is immersed, plotted against the logarithm of the hydrogen ion concentration. (After Klopsteg.)

Fig. 39.—Showing the connections for measuring gas-chain voltage. The E.M.F. from the gas chain is balanced by adjustment of the potential divider until the galvanometer shows zero. The voltmeter, permanently connected as shown, then reads the potential difference which was required to balance the unknown E.M.F. (After Klopsteg.)

the electrode potential of the hydrogen electrode with reference to the solution in which it is immersed, when plotted against the negative logarithm of the hydrogen ion concentration of the solution.

It is usually more convenient to alter the arrangement from that shown in Fig. 37 where two hydrogen electrodes are employed, by replacing one of the hydrogen electrodes with a calomel electrode (the so-called calomel half cell) which can be made up to have a fixed potential. Such an arrangement is shown in Fig. 39. Formula (40) may be rewritten in

terms of one hydrogen electrode immersed in a solution containing a normal concentration of hydrogen ions as a reference electrode, and an electrode immersed in a solution containing an unknown concentration of hydrogen ions (C_a), the concentration of which it is desirable to determine. The formula then becomes

$$E = 0.00019837\ T \log \frac{1}{C_a} \qquad (41)\ ^{13}$$

providing that the concentration of hydrogen ions in the solution of C_a is less than a normal solution. It is very difficult to prepare a solution *exactly* normal with respect to hydrogen ions, and while such a solution has been used as the ultimate standard, the practical reference electrode is the so-called calomel electrode. Formula (41) therefore becomes when the calomel electrode is used,

$$\frac{\text{E.M.F. (observed)} - E\text{ (calomel electrode)}}{0.00019837T} = \log \frac{1}{[H^+]} = p\text{H} \qquad (42)$$

which gives the hydrogen ion concentration directly in pH.

Schmidt and Hoagland[14] have placed in convenient form the pH, C_H, and C_{OH} values corresponding to the various millivolt readings obtained when the normal calomel or the $\frac{N}{10}$ calomel electrode is used as the reference standard. Their values have been calculated by substituting the appropriate values in the above formula.

Lewis, Brighton, and Sebastion[15] give the value of $E = -0.2828$, as the potential of the normal calomel electrode.

Figures 37, 38, and 39 are taken from a paper by Klopsteg[16] where an excellent discussion of the principles involved in the electrometric measurement of hydrogen ion concentration is given. McClendon[17] has also presented an excellent discussion of the principles involved in such measurements, with particular reference to biological applications.

Electrodes other than the normal calomel electrode may be employed

[13] In this equation the logarithm is referred to the base, 10.

[14] Schmidt, C. L. A., and Hoagland, D. R., Table of P_H, H^+, and OH^- Values Corresponding to Electromotive Forces Determined in Hydrogen Electrode Measurements, with a Bibliography, *Univ. of California Publications in Physiol.*, 5 (No. 4): 23–69 (1919).

[15] Lewis G. N., Brighton, T. B., and Sebastion, R. L., A Study of Hydrogen and Calomel Electrodes, *J. Am. Chem. Soc.*, 39 : 2245–2261 (1917).

[16] Klopsteg, P. E., Some Practical Aspects of Hydrogen Electrode Measurements, *J. Ind. Eng. Chem.*, 14 : 399–406 (1922).

[17] McClendon, J. F., Hydrogen and Hydroxyl Ion Concentration in Physiology and Medicine, *Med. Rev. of Rev.*, 22 : 333–365 (1916).

advantageously in certain instances. Gerke[18] has recently published a summary of electrode potentials, and Sharp and MacDougall[19] have described the preparation of a series of electrodes which can be used in titrating solutions to definite *p*H values. Figure 40, taken from their paper, indicates the set-up which is involved. (*B*) is a dipping hydrogen electrode; siphon (*C*) and vessel (*D*) contain a saturated solution of potassium chloride. The liquid to be titrated is placed in beaker (*A*) and connection is made with the reference electrode (*G*), as shown in the diagram. Acid or alkali is added from a burette to the liquid in beaker (*A*) until no deflection is noted on the galvanometer or electrometer, *i.e.*, until no current flows between the reference electrode and the hydrogen electrode. When such an equilibrium is reached, the liquid in beaker (*A*) is at a definite *p*H value depending upon the reference electrode which is used at (*G*). Table XII, taken from their paper, gives the composition of their reference electrodes. The last column in the table indicates the *p*H of the solution in beaker (*A*) at the time that no current flows through the galvanometer.

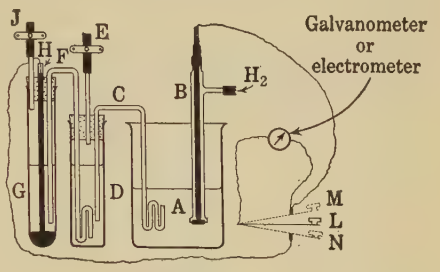


FIG. 40.—Showing the connections necessary to determine the *p*H of an unknown system, using the special electrode half-cells of Sharp and MacDougall.

BUFFERS.—Van Slyke[20] has defined buffers as "*substances which by their presence in solution increase the amount of acid or alkali that must be added to cause unit change in* pH." The most efficient buffers are mixtures of weak acids or weak bases with their corresponding salts.

As noted above, a weak acid or a weak base is characterized by the fact that a large proportion of the material in solution is present in the form of undissociated molecules, and accordingly the dissociation constant is low. In the case of acetic acid only a small fraction of the potential acidity is present at any one time, the dissociation constant

[18] Gerke, R. H., A Summary of Electrode Potentials, *Chem. Rev.*, 1 : 377–395 (1925).

[19] Sharp, P. F., with MacDougall, F. H., A Simple Method of Electrometric Titration in Acidimetry and Alkalimetry, *J. Am. Chem. Soc.*, 44 : 1193–1196 (1922).

[20] Van Slyke, D. D., On the Measurement of Buffer Values and on the Relationship of Buffer Value to the Dissociation Constant of the Buffer and the Concentration and Reaction of the Buffer Solution, *J. Biol. Chem.*, 52 : 525–570 (1922).

TABLE XII

Showing the Composition of Special Electrodes to be Used as Half-Cells in Titrating Solutions to Definite *p*H Values

Electrode Solution. Dilute Quantities Given to Total Volume of 100 cc.				Potential against Normal Calomel Electrode	Calculated Potential of Hydrogen Electrode in Solution of Indicated *p*H	*p*H
	cc.	N	Salt			
12–12.5% Lead Amalgam and Lead Iodide	0.52	2.0	KI	0.5195	0.5194	4.0
	2.90	2.0	KI	0.5609	0.5608	4.7
	6.14	2.0	KI	0.5786	0.5786	5.0
	67.50	2.0	KI	0.6378	0.6377	6.0
12–12.5% Cadmium Amalgam	100.00	0.5	$CdSO_4$	0.6967	0.6968	7.0
	0.20	2.0	KI			
	10.00	0.5	$CdSO_4$	0.7560	0.7560	8.0
	10.14	2.0	KI			
	100.00	0.5	$CdSO_4$	0.8150	0.8151	9.0
	26.40	2.0	KI			
	2.00	0.5	$CdSO_4$	0.8742	0.8743	10.0
	48.70	2.0	KI			

having a value of approximately 1.8×10^{-5}. The equilibrium equation can therefore be written,

$$\frac{[H^+] \times [C_2H_3O_2^-]}{[HC_2H_3O_2]} = 1.8 \times 10^{-5}.$$

The value 1.8×10^{-5} is constant regardless of the concentration of acetic acid in the solution. If more acetic acid is added to the solution, the concentration of undissociated molecules is increased and a small part of the added acetic acid dissociates to maintain the above equilibrium. If another substance with a common ion, for example, sodium acetate, is added, the concentration of the added acetate ions must be taken into consideration in the above equation, and since this would cause an increase in the anion portion of the numerator, there would be a corresponding decrease in the hydrogen ion concentration.

Buffers may also be defined as *those substances which prevent sudden or great changes in hydrogen ion concentration when strong acids or bases*

are added to a system. If one adds hydrochloric acid to a solution of tri-sodium phosphate, the following reactions take place,

$$Na_3PO_4 + HCl = NaCl + Na_2HPO_4;$$
$$Na_2HPO_4 + HCl = NaCl + NaH_2PO_4;$$
$$NaH_2PO_4 + HCl = NaCl + H_3PO_4.$$

Assuming that the original solution of tri-sodium phosphate is a 0.3N solution and that one equivalent of hydrochloric acid is added to it, the solution of di-sodium phosphate which results from the adding of the strongly acid hydrochloric acid (pH = 1) would not only be not acid but would in reality be alkaline and would have a pH of approximately 8.5 to 9.

If to this slightly alkaline solution, we again add an equivalent of hydrochloric acid, the resulting mono-sodium phosphate solution would have a pH of approximately 4.5, and if to this solution of mono-sodium phosphate a third equivalent of hydrochloric acid is added, the resulting solution of phosphoric acid would have a pH of approximately 2.5. Three equivalents of hydrochloric acid, having a pH of 1.0, have thus been needed to shift the hydrogen ion concentration of tri-sodium phosphate from approximately pH = 10.5 to approximately pH = 2.5.

In a similar way the reaction between sodium acetate and hydrochloric acid may be written,

$$NaC_2H_3O_2 + HCl = NaCl + H \cdot C_2H_3O_2$$

and in this case the hydrochloric acid solution having a pH of 1.0 has been "buffered" by the sodium acetate, so that the resulting mixture has a pH of approximately 2.5. Sodium carbonate, likewise, acts as a buffer according to the following reaction,

$$Na_2CO_3 + HCl = NaCl + NaHCO_3,$$

and on the addition of a second equivalent of hydrochloric acid,

$$NaHCO_3 + HCl = NaCl + H_2CO_3.$$

Carbonic acid is such a weak acid that it decomposes according to the following equation,

$$H_2CO_3 = CO_2 + H_2O,$$

and the carbon dioxide may be given off from the solution in the form of gas bubbles. Thus, we have here conditions where two equivalents of a strong acid can be added to a slightly alkaline solution, with a resulting solution which is essentially neutral. *This is one of the reactions by which the animal body is protected against sudden changes in hydrogen ion*

concentration. The blood contains a considerable amount of bicarbonate. If diluted hydrochloric acid is injected intravenously, the acid is buffered by the bicarbonate of the blood stream; the carbon dioxide which is formed is eliminated by the lungs; the hydrochloric acid is converted into sodium chloride, a normal constituent of the blood; a part of the remaining bicarbonate dissociates so as to keep a constant K_b; and there is no appreciable change in the hydrogen ion concentration of the blood stream. Very considerable amounts of strong acids can accordingly be neutralized by the body through the action of the normal buffers of the blood and tissues. If, however, we were to continue the injection of dilute hydrochloric acid until the buffers in the blood stream had been exhausted, there would suddenly occur a marked rise in the hydrogen ion concentration of the blood, a violent physiological reaction, and if sufficient hydrochloric acid were injected, death would ensue. *All reactions of living protoplasm take place in buffered media.* Carbonates, bicarbonates, and phosphates are the principal buffers met with in biological processes, although proteins may under certain conditions act as relatively inefficient buffers.

The same sort of reactions which take place in the buffering of a strong acid are involved when strong bases are employed. Thus, for example, ammonium acetate will act as a buffer for sodium hydroxide according to the following reaction,

$$NH_4C_2H_3O_2 + NaOH = NaC_2H_3O_2 + NH_4OH,$$

where the strong base, sodium hydroxide, has been buffered and replaced by the weak ammonium hydroxide, or using a phosphate,

$$NaH_2PO_4 + NaOH = Na_2HPO_4 + H_2O.$$

Most biological reactions take place in an essentially neutral or slightly acid medium, and as a rule biological organisms have a greater capacity for the buffering of acid solutions than they have for the buffering of bases.

Van Slyke[21] has proposed a unit for the measurement of buffering values. The unit adopted is the differential ratio, $\frac{dB}{dp\mathrm{H}}$, which expresses the relationship between the increment in gram equivalents per liter of a strong base (B) added to a buffer solution, and the resultant increment in pH. Correspondingly for the acid range the increment of strong acid is equivalent to a negative increment of the base ($-dB$). In these terms

[21] Van Slyke, D. D., On the Measurement of Buffer Values and on the Relationship of Buffer Value to the Dissociation Constant of the Buffer and the Concentration and Reaction of the Buffer Solution, *J. Biol. Chem.*, 52 : 525–570 (1922).

a solution has a buffer value of 1.0 when a liter will take up a gram equivalent of strong acid or alkali per unit change in pH.

As Van Slyke points out, if a base is added to a solution, the pH is increased so that both dB and dpH are positive. If an acid is added, dB and dpH both are negative. However, the ratio $\frac{dB}{dp\text{H}}$ always has a positive numerical value. If one solution has twice the buffer value of a

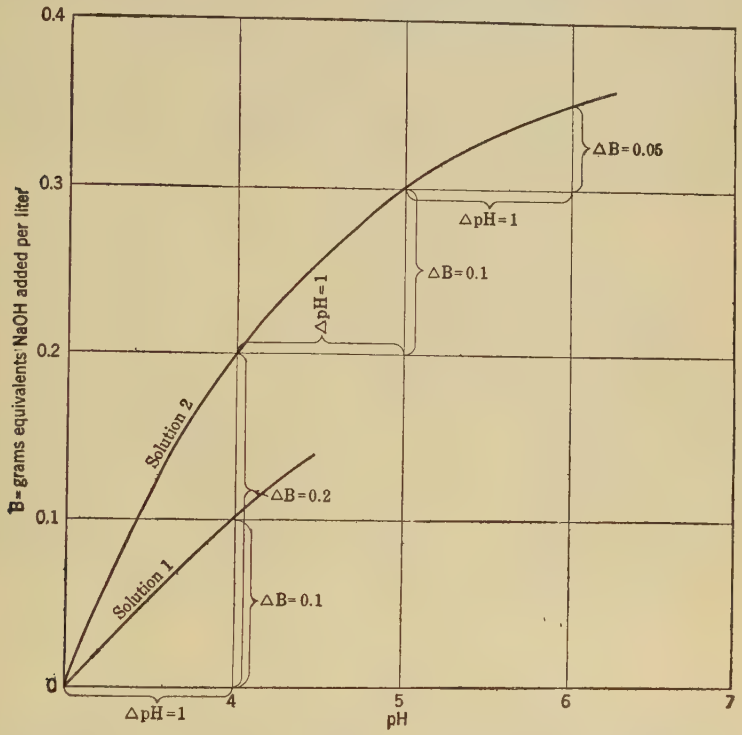


FIG. 41.—Showing the relationship between buffer capacity (ΔB) and change in hydrogen ion concentration. (Data of Van Slyke.)

second solution, it will require twice as much acid or base to change the pH of the former through a unit range. The value of $\frac{dB}{dp\text{H}}$ is therefore twice as great for the first solution as for the second. Van Slyke suggests that the symbol β be used to indicate the ratio $\frac{dB}{dp\text{H}}$. Figure 41, taken from the paper by Van Slyke, represents the buffer value of the two solutions referred to in this paragraph.

It would take us too far afield to consider adequately the various factors which must be taken into consideration in a study of buffer action. The manuals by Clark and Michaelis are adequate for a general understanding of the principles which are involved, and the paper by Van Slyke which is noted above gives an extended discussion of the theories which are involved. The average biochemist or biologist is usually concerned not so much with the theory underlying the general principle as he is with the practical application of the principle to laboratory problems. Perhaps the greatest application of the principles of buffer action aside from the interpretation of the resistance of living organisms to changes in acidity or alkalinity, lies in the ability to prepare from known mixtures of chemicals, solutions which have a definite and stable *p*H value. Table XIII lists the composition of buffer mixtures having a given *p*H value at 20° C.

TABLE XIII

Composition of Mixtures Giving *p*H Values at 20° C. at Intervals of 0.2 *

KCl–HCl Mixtures †

*p*H			
1.2	50 cc. M/5 KCl	65.5 cc. M/5 HCl	Dilute to 200 cc.
1.4	50 cc. M/5 KCl	41.5 cc. M/5 HCl	Dilute to 200 cc.
1.6	50 cc. M/5 KCl	26.3 cc. M/5 HCl	Dilute to 200 cc.
1.8	50 cc. M/5 KCl	16.6 cc. M/5 HCl	Dilute to 200 cc.
2.0	50 cc. M/5 KCl	10.6 cc. M/5 HCl	Dilute to 200 cc.
2.2	50 cc. M/5 KCl	6.7 cc. M/5 HCl	Dilute to 200 cc.

Phthalate–HCl Mixtures

2.2	50 cc. M/5 KH Phthalate	46.70 cc. M/5 HCl	Dilute to 200 cc.
2.4	50 cc. M/5 KH Phthalate	39.60 cc. M/5 HCl	Dilute to 200 cc.
2.6	50 cc. M/5 KH Phthalate	32.95 cc. M/5 HCl	Dilute to 200 cc.
2.8	50 cc. M/5 KH Phthalate	26.42 cc. M/5 HCl	Dilute to 200 cc.
3.0	50 cc. M/5 KH Phthalate	20.32 cc. M/5 HCl	Dilute to 200 cc.
3.2	50 cc. M/5 KH Phthalate	14.70 cc. M/5 HCl	Dilute to 200 cc.
3.4	50 cc. M/5 KH Phthalate	9.90 cc. M/5 HCl	Dilute to 200 cc.
3.6	50 cc. M/5 KH Phthalate	5.97 cc. M/5 HCl	Dilute to 200 cc.
3.8	50 cc. M/5 KH Phthalate	2.63 cc. M/5 HCl	Dilute to 200 cc.

* From W. M. Clark, "The Determination of Hydrogen Ions," Williams and Wilkins Company, Baltimore. 1928. By permission.

† The *p*H values of these mixtures are given by Clark and Lubs (1916) as *preliminary* measurements.

TABLE XIII—*Continued*

Phthalate–NaOH Mixtures

*p*H			
4.0	50 cc. M/5 KH Phthalate	0.40 cc. M/5 NaOH	Dilute to 200 cc.
4.2	50 cc. M/5 KH Phthalate	3.70 cc. M/5 NaOH	Dilute to 200 cc.
4.4	50 cc. M/5 KH Phthalate	7.50 cc. M/5 NaOH	Dilute to 200 cc.
4.6	50 cc. M/5 KH Phthalate	12.15 cc. M/5 NaOH	Dilute to 200 cc.
4.8	50 cc. M/5 KH Phthalate	17.70 cc. M/5 NaOH	Dilute to 200 cc.
5.0	50 cc. M/5 KH Phthalate	23.85 cc. M/5 NaOH	Dilute to 200 cc.
5.2	50 cc. M/5 KH Phthalate	29.95 cc. M/5 NaOH	Dilute to 200 cc.
5.4	50 cc. M/5 KH Phthalate	35.45 cc. M/5 NaOH	Dilute to 200 cc.
5.6	50 cc. M/5 KH Phthalate	39.85 cc. M/5 NaOH	Dilute to 200 cc.
5.8	50 cc. M/5 KH Phthalate	43.00 cc. M/5 NaOH	Dilute to 200 cc.
6.0	50 cc. M/5 KH Phthalate	45.45 cc. M/5 NaOH	Dilute to 200 cc.
6.2	50 cc. M/5 KH Phthalate	47.00 cc. M/5 NaOH	Dilute to 200 cc.

KH_2PO_4–NaOH Mixtures

*p*H			
5.8	50 cc. M/5 KH_2PO_4	3.72 cc. M/5 NaOH	Dilute to 200 cc.
6.0	50 cc. M/5 KH_2PO_4	5.70 cc. M/5 NaOH	Dilute to 200 cc.
6.2	50 cc. M/5 KH_2PO_4	8.60 cc. M/5 NaOH	Dilute to 200 cc.
6.4	50 cc. M/5 KH_2PO_4	12.60 cc. M/5 NaOH	Dilute to 200 cc.
6.6	50 cc. M/5 KH_2PO_4	17.80 cc. M/5 NaOH	Dilute to 200 cc.
6.8	50 cc. M/5 KH_2PO_4	23.65 cc. M/5 NaOH	Dilute to 200 cc.
7.0	50 cc. M/5 KH_2PO_4	29.63 cc. M/5 NaOH	Dilute to 200 cc.
7.2	50 cc. M/5 KH_2PO_4	35.00 cc. M/5 NaOH	Dilute to 200 cc.
7.4	50 cc. M/5 KH_2PO_4	39.50 cc. M/5 NaOH	Dilute to 200 cc.
7.6	50 cc. M/5 KH_2PO_4	42.80 cc. M/5 NaOH	Dilute to 200 cc.
7.8	50 cc. M/5 KH_2PO_4	45.20 cc. M/5 NaOH	Dilute to 200 cc.
8.0	50 cc. M/5 KH_2PO_4	46.80 cc. M/5 NaOH	Dilute to 200 cc.

Boric Acid. KCl–NaOH Mixtures

*p*H			
7.8	50 cc. M/5 H_3BO_3, M/5 KCl	2.61 cc. M/5 NaOH	Dilute to 200 cc.
8.0	50 cc. M/5 H_3BO_3, M/5 KCl	3.97 cc. M/5 NaOH	Dilute to 200 cc.
8.2	50 cc. M/5 H_3BO_3, M/5 KCl	5.90 cc. M/5 NaOH	Dilute to 200 cc.
8.4	50 cc. M/5 H_3BO_3, M/5 KCl	8.50 cc. M/5 NaOH	Dilute to 200 cc.
8.6	50 cc. M/5 H_3BO_3, M/5 KCl	12.00 cc. M/5 NaOH	Dilute to 200 cc.
8.8	50 cc. M/5 H_3BO_3, M/5 KCl	16.30 cc. M/5 NaOH	Dilute to 200 cc.
9.0	50 cc. M/5 H_3BO_3, M/5 KCl	21.30 cc. M/5 NaOH	Dilute to 200 cc.
9.2	50 cc. M/5 H_3BO_3, M/5 KCl	26.70 cc. M/5 NaOH	Dilute to 200 cc.
9.4	50 cc. M/5 H_3BO_3, M/5 KCl	32.00 cc. M/5 NaOH	Dilute to 200 cc.
9.6	50 cc. M/5 H_3BO_3, M/5 KCl	36.85 cc. M/5 NaOH	Dilute to 200 cc.
9.8	50 cc. M/5 H_3BO_3, M/5 KCl	40.80 cc. M/5 NaOH	Dilute to 200 cc.
10.0	50 cc. M/5 H_3BO_3, M/5 KCl	43.90 cc. M/5 NaOH	Dilute to 200 cc.

Sörensen[22] gives a series of buffer mixtures different from those suggested by Clark. The compositions of Sörensen's mixtures are shown in Table XIV.

TABLE XIV

The Composition of Sörensen's Buffer Mixtures

(*a*) Mixtures of Glycine * and Hydrochloric Acid †

Volume of Glycine Solution	Volume of HCl	*p*H of Mixture	Volume of Glycine Solution	Volume of HCl	*p*H of Mixture
cc.	cc.		cc.	cc.	
10.00	.....	6.106	6.00	4.00	2.279
9.90	0.10	4.411	5.00	5.00	1.932
9.75	0.25	3.991	4.00	6.00	1.645
9.50	0.50	3.679	3.00	7.00	1.419
9.00	1.00	3.341	2.00	8.00	1.251
8.00	2.00	2.922	1.00	9.00	1.146
7.00	3.00	2.607	.....	10.00	1.038

* 7.505 grams of glycine and 5.85 grams NaCl in one liter of solution.

†An exactly 0.1 N solution of hydrochloric acid.

(*b*) Mixtures of Glycine * and Sodium Hydroxide †

Volume of Glycine Solution	Volume of NaOH	*p*H of Mixture	Volume of Glycine Solution	Volume of NaOH	*p*H of Mixture
cc.	cc.		cc.	cc.	
10.00	.....	6.106	5.10	4.90	11.067
9.90	0.10	7.809	5.00	5.00	11.305
9.75	0.25	8.237	4.90	5.10	11.565
9.50	0.50	8.575	4.50	5.50	12 095
9.00	1.00	8.929	4.00	6.00	12.399
8.00	2.00	9.364	3.00	7.00	12.674
7.00	3.00	9.714	2.00	8.00	12.856
6.00	4.00	10.140	1.00	9.00	12.972
5.50	4.50	10.482	.....	10.00	13.066

* Same concentration as in Section (*a*) of this table.

† An exactly 0.1 N solution of carbonate-free NaOH.

[22] Sörensen, S. P. L., Études enzymatiques. II. Sur la mesure et l'importance de la concentration des ions hydrogène dans les réactions enzymatiques, *Compt. rend. Lab. Carlsberg*, 8 : 41, 42, 43 (1909). Compare also Kolthoff, I. M., A New Set of Buffer Mixtures That Can Be Prepared Without the Use of Standardized Acid or Base, *J. Biol. Chem.*, 63 : 135–141 (1925).

TABLE XIV—*Continued*

(*c*) Mixtures of Sodium Monohydrogen Phosphate * and Potassium Dihydrogen Phosphate †

Volume of Na_2HPO_4 Solution	Volume of KH_2PO_4 Solution	*p*H of Mixture	Volume of Na_2HPO_4 Solution	Volume of KH_2PO_4 Solution	*p*H of Mixture
cc.	cc.		cc.	cc.	
10.00	.....	8.302	4.00	6.00	6.643
9.90	0.10	8.171	3.00	7.00	6.468
9.75	0.25	8.038	2.00	8.00	6.239
9.50	0.50	7.863	1.00	9.00	5.910
9.00	1.00	7.648	0.50	9.50	5.600
8.00	2.00	7.347	0.25	9.75	5.305
7.00	3.00	7.146	0.10	9.90	4.976
6.00	4.00	6.976	.....	10.00	4.529
5.00	5.00	6.813			

* 11.876 grams $Na_2HPO_4 \cdot 2H_2O$ in one liter of solution.
† 9.078 grams KH_2PO_4 in one liter of solution.

(*d*) Mixtures of Sodium Borate * and 0.1 N Hydrochloric Acid

Volume of Borate Solution	Volume of HCl	*p*H of Mixture	Volume of Borate Solution	Volume of HCl	*p*H of Mixture
cc.	cc.		cc.	cc.	
10.00	.....	9.241	6.50	3.50	8.506
9.50	0.50	9.168	6.00	4.00	8.289
9.00	1.00	9.087	5.75	4.25	8.137
8.50	1.50	9.007	5.50	4.50	7.939
8.00	2.00	8.908	5.25	4.75	7.621
7.50	2.50	8.799	5.00	5.00	6.548
7.00	3.00	8.678	4.75	5.25	2.371

* 12.404 grams of boric acid dissolved in 100 cc. of exactly 1.0 N carbonate-free NaOH and diluting the mixture to 1 liter volume.

(*e*) Mixtures of Sodium Borate * and 0.1 N Sodium Hydroxide

Volume of Borate Solution	Volume of NaOH	*p*H of Mixture	Volume of Borate Solution	Volume of NaOH	*p*H of Mixture
cc.	cc.		cc.	cc.	
10.00	.....	9.241	6.00	4.00	9.974
9.00	1.00	9.360	5.00	5.00	11.076
8.00	2.00	9.503	4.00	6.00	12.376
7.00	3.00	9.676			

* Borate solution of same composition as in Section (*d*) of this table.

TABLE XIV—*Continued*

(*f*) Mixture of Sodium Citrate * and 0.1 N Hydrochloric Acid

Volume of Citrate Solution	Volume of HCl	*p*H of Mixture	Volume of Citrate Solution	Volume of HCl	*p*H of Mixture
cc.	cc.		cc.	cc.	
10.00	.....	4.958	4.75	5.25	3.529
9.50	0.50	4.887	4.50	5.50	3.364
9.00	1.00	4.830	4.00	6.00	2.972
8.00	2.00	4.652	3.33	6.67	2.274
7.00	3.00	4.447	3.00	7.00	1.925
6.00	4.00	4.158	2.00	8.00	1.418
5.50	4.50	3.948	1.00	9.00	1.173
5.00	5.00	3.692	.....	10.00	1.038

* 21.008 grams of citric acid ($C_6H_8O_7 \cdot H_2O$) dissolved in 200 cc. of carbonate-free 1.0 N NaOH solution and the mixture diluted to 1 liter.

(*g*) Mixtures of Sodium Citrate * and 0.1 N Sodium Hydroxide

Volume of Citrate Solution	Volume of NaOH	*p*H of Mixture	Volume of Citrate Solution	Volume of NaOH	*p*H of Mixture
cc.	cc.		cc.	cc.	
10.00	.....	4.958	5.50	4.50	6.331
9.50	0.50	5.023	5.25	4.75	6.678
9.00	1.00	5.109	5.00	5.00	9.052
8.00	2.00	5.314			10.092
7.00	3.00	5.568	4.50	5.50	12.073
6.00	4.00	5.969	4.00	6.00	12.364

* Same concentration as solution used in Section (*f*) of this table.

As noted above, proteins may act to a certain extent as buffers. Their efficiency as buffers undoubtedly depends to a very great extent on the chemical nature of the amino acids making up the protein molecule. Figure 42, taken from the data of Hoffman and Gortner[23] shows the change in hydrogen ion concentration of two typical proteins when acid or alkali is added to the system. In the case of durumin, the prolamine of durum wheat, there is no evidence of buffer action toward sodium

[23] Hoffman, W. F., and Gortner, R. A., Physico-chemical Studies on Proteins. I. The Prolamines—Their Chemical Composition in Relation to Acid and Alkali Binding, Colloid Symposium Monograph, Vol. II, pp. 209–368, Chemical Catalog Company, New York (1925).

hydroxide, and not much evidence of buffer action toward hydrochloric acid. In the case of casein from milk there is a definite buffering of added sodium hydroxide in the range from approximately *p*H 6.0 to *p*H 7.0, with a slight but less pronounced buffering effect against acid in

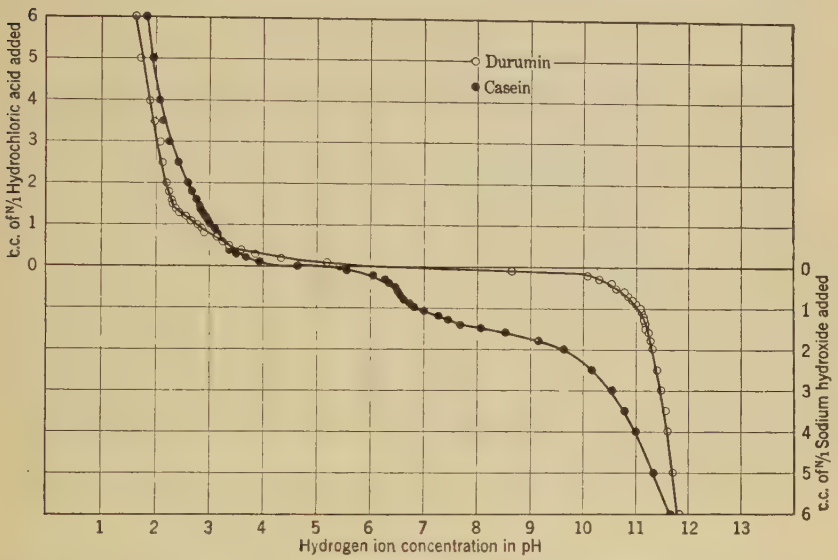


FIG. 42.—Buffer curves of durumin and casein in various concentrations of hydrochloric acid and sodium hydroxide. (Data of Hoffman and Gortner.)

the range of approximately *p*H 4.0 to *p*H 3.0. On the whole, however, proteins may be regarded as fairly inefficient buffers, and the generalization noted above that carbonates, bicarbonates, and phosphates are the principal buffers of biological systems still holds.

CHAPTER V

ELECTROKINETIC PHENOMENA OF COLLOID SYSTEMS

FREUNDLICH[1, 2] has designated certain electrical properties of colloid systems by the term electrokinetic phenomena in order to distinguish them from a similar, although not identical, electrical phenomenon known as the thermodynamic potential which exists at interfaces. As Freundlich points out, the electrokinetic phenomena are very closely correlated with many physical properties of colloid systems. As we shall see later, *the phenomena of adsorption, interfacial tension, mutual precipitation, flocculation, colloid stability, and the behavior of a colloid system under the influence of an electric current are all related to the so-called electrokinetic phenomena.*

We have already noted (Chapter II) that the micelles present in colloidal systems possess either a positive or a negative electrical charge and that as this charge becomes reduced to approximately zero, the colloid system becomes unstable. Accordingly a consideration of the nature of this charge and of the methods for the determination of its exact magnitude is of great importance.

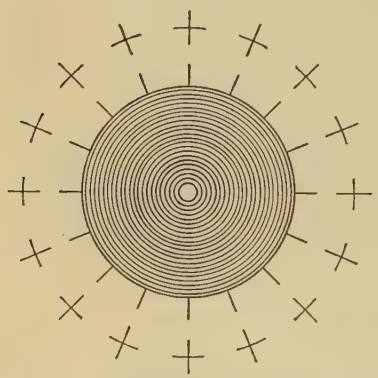


FIG. 43.—A diagrammatic representation of the distribution of the electrical charges in a "Helmholtz double layer" surrounding a micelle.

All of the theories which have been advanced to explain electrokinetic phenomena are based upon the theory of Helmholtz, involving the presence or absence of an electric double layer in the immediate vicinity of the colloid micelle. Figure 43 is a diagrammatic representation of a colloidal micelle surrounded by the Helmholtz double layer. In this figure the micelle

[1] Freundlich, H., Colloid and Capillary Chemistry, translated by H. S. Hatfield, E. P. Dutton and Company, New York (1926).

[2] Freundlich, H., New Conceptions in Colloidal Chemistry, E. P. Dutton and Company, New York (1927).

will possess a negative charge, the dispersions medium in its immediate vicinity being positively charged.

We have already noted that an electrical charge on a colloidal micelle may arise from several causes, such as the *direct ionization* of the material comprising the micelle, the *capture of an ion* by a micelle (adsorption), or *electrification by contact.* Michaelis[3] classifies the causes as due to (1) the forces of residual valencies which cause oriented adsorption, (2) the forces of dissociation which cause exchange adsorption, and (3) the spontaneous distribution of ions at a free surface which comes into play in those instances where the electric double layer is set up at the boundary of substances which are incapable of dissociation and which do not react chemically.

In the first instance, silver bromide crystals, for example, contain in their crystal faces both silver and bromine atoms, the charges of which are not completely neutralized. Accordingly, if there is an excess of bromine ions in the surrounding liquid, they will be attracted to the vicinity of the unsaturated silver atoms in the crystal face; if there is an excess of silver ions in the surrounding solution, they will be attracted to the unsaturated bromine atoms in the crystal face. The result has already been shown diagrammatically in Fig. 6. In the event that the bromine ions are in excess, the colloid micelle will be negatively charged, whereas it will be positively charged if the silver ions are in excess. The electric double layer is, therefore, reversed as we pass the isoelectric point. No difference in potential in the double layer will exist at the isoelectric point.

It is easy to picture how dissociation brings about an electric double layer. The dissociation of a ferric hydroxide sol would result in the negative hydroxyl ion being given off into the solution with a corresponding residual positive charge remaining on the micelle. It is obvious that the negative charge cannot be separated by any great distance from the residual positive charge. Accordingly the double layer is set up.

The third class of substances noted by Michaelis comprises such substances as cellulose, collodion, air bubbles, colloidal carbon, hydrocarbons, etc., where there is probably no great tendency to dissociate and a relatively slight tendency toward oriented adsorption. In this instance Michaelis assumes a selective adsorption of either hydrogen or hydroxyl ions from the aqueous phase (dispersions medium). Assuming that OH^- is more capillary active than H^+, certain of the hydroxyl ions of water would enter more closely to the surface of the disperse phase, so

[3] Michaelis, L., The Effects of Ions in Colloidal Systems, Williams and Wilkins Company, Baltimore (1925).

that there would be near the surface of the disperse phase a greater concentration of hydroxyl ions than of hydrogen ions. Accordingly, a double layer would be set up, the micelle acquiring a negative charge.

In a consideration of the theories underlying the quantitative determination of electrokinetic potentials, extensive use has been made of the recent historical review presented by Briggs.[4] Reuss, in 1808, observed that when an electric current was passed through an earthenware diaphragm, water was transferred from the anode to the cathode chamber. Porret, in 1816, described the same phenomenon, using a sand diaphragm. This transfer of water, due to the passage of an electric current through a porous diaphragm, became known as *electroendosmosis*, and quantitative measurements were made by Wiedemann, and by Quincke.[5] Quincke observed that water might travel either toward the anode or toward the cathode, depending on the nature of the solid which was used, and postulated the existence of an electric double layer across the interface, the orientation of the layer being different for different substances. Helmholtz,[6] Perrin,[7] Lamb,[8] and Smoluchowski[9] have expressed the theory of electroendosmosis in mathematical form. Helmholtz developed his equation by assuming a single capillary tube of the solid phase filled with a liquid and placed in an electrical field. He assumed the electrical double layer at the interface to be essentially a condenser, the plates of which were separated by a distance equal to the diameter of one molecule. Perrin introduced into the equation of Helmholtz the dielectric constant of the liquid as an influencing factor.

Let us assume the existence of an electrical double layer on the wall of a capillary, one part of the electrical double layer being fixed to the solid phase, the other portion, located at distance (δ) from the opposite charge, being in the liquid phase and free to move with the liquid. If, now, a potential difference is applied to the ends of the capillary, one charge tends to move toward the anode, the other toward the cathode, but since the charge on the solid is fixed, the charged liquid layer will

[4] Briggs, D. R., The Determination of the ζ-Potential on Cellulose—A Method, *J. Phys. Chem.*, 32 : 641–675 (1928).

[5] Quincke, G., Ueber die Fortführung materieller Theilchen durch strömende Elektricität, *Pogg. Ann.*, 113 : 513–598 (1861).

[6] Helmholtz, H., Studien über elektrische Grenzschten, *Wied. Ann.*, 7 : 337–381 (1879); *Wissenschaftliche Abhandlungen*, 1 : 855–898 (1882).

[7] Perrin, J., Mécanisme de l'électrisation de contact et solutions colloidales, *J. Chim. phys.*, 2 : 601–651 (1904); 3 : 50–110 (1905).

[8] Lamb, H., On the Theory of Electric Endosmosis and Other Allied Phenomena, *Brit. Assoc. Rep.*, 495–510 (1887).

[9] von Smoluchowski, M., Contribution à la théorie de l'endosmose électrique et de quelques phénomènes corrélatifs, *Bull, Acad. Sci. Cracovie*, 182–199 (1903).

move along the surface of the capillary at such a rate that the frictional forces (R) will be at equilibrium with the electrical forces (F). The electrical force (F), acting on a unit area of surface, is equal to the product of the charge (e) on the double layer per unit of surface and the potential difference (E) which is applied to a unit length.

$$F = eE \tag{42}$$

At equilibrium this must be equal to the frictional force which must be overcome or balanced. The frictional force (R) is related to the coefficient of viscosity of the liquid and to the decrease in velocity of the flow of the liquid in a direction perpendicular to the wall of the capillary, *i.e.*, the average velocity of the liquid layer between the point of maximum velocity (u) and the point where no flow takes place, the distance between these two points being designated as (δ). The frictional force can therefore be expressed as

$$R = \frac{\eta u}{\delta} \tag{43}$$

(u) may be evaluated by measuring the volume (V) of liquid flowing in a given time through a known area of cross section of the tube, according to the formula

$$V = \pi r^2 u \tag{44}$$

accordingly,

$$R = \frac{\eta V}{\delta \pi r^2} \tag{45}$$

and from the formula (42),

$$eE = \frac{\eta V}{\delta \pi r^2} \tag{46}$$

On the assumption that the properties of the double layer are those of two plates of a flat condenser, we can state that the capacity (C) of the double layer is directly proportional to the quantity (Q) of electricity which it holds and inversely proportional to the potential difference (ζ) across the plates,

$$C = \frac{Q}{\zeta} \tag{47}$$

The capacity is likewise proportional to the dielectric constant (ϵ) of the material separating the plates, to the area (A) of the plates, and inversely proportional to their distance apart (δ) and to a constant (4π). Accordingly,

$$C = \frac{\epsilon A}{4\pi\delta} \tag{48}$$

therefore,

$$\frac{Q}{\zeta} = \frac{\epsilon A}{4\pi\delta} \tag{49}$$

and,

$$\zeta = \frac{4\pi\delta Q}{\epsilon A} \tag{50}$$

Substituting (e), the charge per unit area, for the quantity $\frac{Q}{A}$, we have

$$\zeta = \frac{4\pi\delta e}{\epsilon} \quad \text{or} \quad \pi\delta = \frac{\zeta\epsilon}{4e} \tag{51}$$

Again substituting, we have

$$eE = \frac{\eta V 4e}{\zeta\epsilon r^2} \tag{52}$$

Accordingly,

$$V = \frac{r^2\zeta E\epsilon}{4\eta} \tag{53}$$

and

$$\zeta = \frac{4V\eta}{r^2 E\epsilon} \tag{54}$$

However, in a diaphragm the πr^2 of a capillary tube must be replaced by the area of cross section (q), r^2 equalling $\frac{q}{\pi}$. Accordingly,

$$V = \frac{q\zeta E\epsilon}{4\pi\eta} \tag{55}$$

and

$$\zeta = \frac{4\pi V\eta}{qE\epsilon} \tag{56}$$

(E) is equal to (H), the E.M.F. applied across the diaphragm, divided by (l), the distance between the ends of the diaphragm. Likewise, (H), the applied E.M.F., is equal to (i), the current, multiplied by (w), the resistance, where (w) is equal to the distance, (l), between the ends of the diaphragm, divided by the product of (q), the cross section area, and (κ), the specific conductivity of the liquid. Therefore,

$$V = \frac{\zeta i\epsilon}{4\pi\eta\kappa} \tag{57}$$

and

$$\zeta = \frac{4\pi\eta\kappa V}{i\epsilon} \tag{58}$$

This equation states that where the electric current (i) is kept constant, the volume of liquid (V) which will flow in a given time through a diaphragm is directly proportional to the ζ-potential across the interface, to the dielectric constant (ϵ) of the liquid, and inversely proportional to the viscosity (η), and the specific conductivity of the liquid (κ) and is independent of the area of cross section or the length of the capillary.

The derivation of this formula has been given at length because of the fact that methods based upon this formula promise to be of great service in colloid studies involving biochemical materials. Briggs has adequately discussed the various assumptions upon which this formula is based. He points out that the formula is probably not strictly accurate in that it assumes a dielectric constant of 80 for water in the immediate vicinity of the colloid micelle, whereas in all probability the compressed water layer on the surface of the micelle has a lower dielectric constant. Likewise, in the above derivation, two distances (δ) have been evaluated as equal, while as a matter of fact they are probably not equal. It is likewise highly probable that (δ) the distance between the charges in the Helmholtz double layer may not be a fixed quantity but may be a variable quantity.

McClendon[10] points out that the strength of the solution in contact with the interface causes a variation in the thickness of the double layer. Thus, in 0.1 N NaCl solution he found a value of $1.94m\mu$ for the thickness of the double layer which increased to $3.25m\mu$ when the solution of NaCl was 0.001 N. Gouy[11] calculated that this must be true and obtained theoretical values of $0.96m\mu$ for a 0.1 N solution and $9.6m\mu$ for a 0.001 N solution, and for pure water (conductivity water) a value of $1010m\mu$, assuming a dielectric constant of 80.

Briggs likewise points out that the specific electrical conductivity (κ) is not the specific electrical conductivity of the liquid which is passing through the pores of the diaphragm but rather is the specific conductivity of the liquid in the diaphragm. Accordingly formula (58) should be rewritten by substituting (κ_s), the specific conductivity of the liquid within the diaphragm, for the (κ), the specific conductivity of the pure liquid.

The quantity (ζ) in equation (58) represents the absolute electrical charge at an interface as nearly as it can be evaluated at the present time.

As noted in the opening paragraph of this chapter, the electrokinetic

[10] McClendon, J. F., On the Thickness of the Helmholtz Double Layer, *Science*, 66 : 200 (1927).

[11] Gouy, M., Sur la constitution de la charge électrique à la surface d'un électrolyte, *J. de Phys.* (4) 9 : 457–468 (1910).

potential is distinct from the Nernst thermodynamic potential or the boundary potential which exists across interfaces. We have already noted in the consideration of hydrogen ion concentration that a difference in potential exists at the boundary of two solutions which differ in concentration. This boundary potential can be stated as

$$\epsilon_b = -\frac{RT}{F} \log_e \frac{C_1}{C_2} \tag{59}$$

where ϵ_b = the boundary potential;
R = the gas constant;
T = absolute temperature;
F = the faraday;
C_1 and C_2 = the respective concentrations of ions in the two solutions.

The importance of this equation in biochemical problems will be evident when we consider the Donnan equilibrium (*vide infra*).

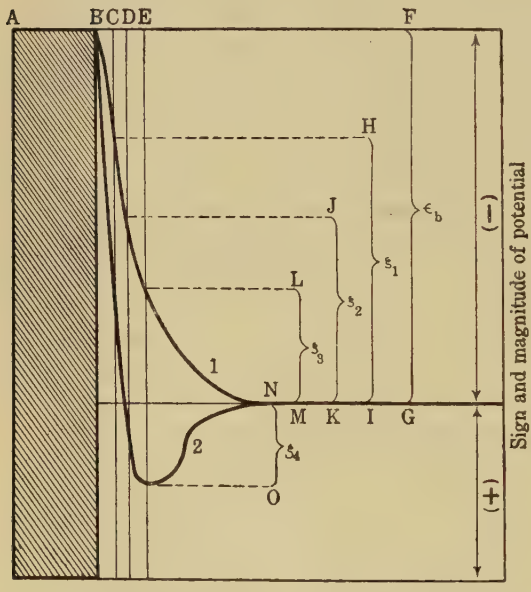


FIG. 44. A diagrammatic representation of the possible relationship between the thermodynamic or Nernst potential and the electrokinetic or ζ-potential (for explanation see text).

Figure 44 illustrates in a diagrammatic way the relationships which may exist between (ζ), the electrokinetic potential and (ϵ_b) the thermodynamic or Nernst potential, and shows how it is possible to conceive of the electrokinetic potential varying both in magnitude and in sign, while the thermodynamic potential remains constant.

Let the cross-hatched section between the lines (A) and (B) at the left-hand of Fig. 44 represent a colloid gel in equilibrium with a liquid phase. The thermodynamic potential (ϵ_b) would be the potential which was measured from some point on the interior of the gel to some point well within the interior of the surrounding medium. The magnitude of this potential is represented on the diagram by the line (FG).

The electrokinetic potential, however, has already been defined as the potential which exists between the movable and immovable liquid layers at the interface. In other words, the interface which gives rise to the potential (ϵ_b) is the interface of gel-liquid. The interface which gives rise to (ζ) is the interface between the immovable liquid film adhering to the surface of the gel and the body of the liquid. Curve (1), Fig. 44, represents the hypothetical fall in electrokinetic potential from the surface of the gel into the body of the liquid. Curve (2) represents a second hypothetical fall of the electrokinetic potential. As we shall see later when we discuss the effect of ions on colloid systems, curves similar to both (1) and (2) have been demonstrated experimentally.

With a gradient of electrokinetic potential, such as is postulated in curve (1), it should be possible to have values of (ζ) which vary in magnitude from (ζ_1) to (ζ_2) to (ζ_3), depending on the thickness of the immovable, adsorbed water layer adhering to the surface of the gel, assuming that the ion concentration in the immovable, adsorbed water layer varies with the thickness of the bound-water film, *i.e.*, that ions and water molecules are not necessarily adsorbed in the same ratio. We shall see when we discuss the solvation of lyophilic colloids that the thickness of the water film adsorbed to the surface of the colloid particle may vary within wide limits. As a matter of fact, we have already noted this variation, for the viscosity curves shown in Figs. 14, 15, 34, and 35 represent in reality changes in the thickness of the water film associated with the disperse phase. Accordingly if the hypothetical water film in Fig. 44 has a thickness (BC), the boundary between the movable and immovable phases would occur at (C), and (ζ_1) would represent the magnitude of the electrokinetic potential. If, however, the water film had a thickness (BD), the movable boundary would be shifted to (D) and the magnitude of (ζ_2) would measure the electrokinetic potential. If the water film were further extended to (E), so that (E) became the boundary between the movable and immovable portions, the electrokinetic potential would be equivalent to (ζ_3). Assuming the potential gradient to fall to curve (2) and assuming line (E) to represent the boundary between the movable and the immovable layers, the electrokinetic potential curve would cut this line at point (0), and the electrokinetic potential produced would be equivalent to (ζ_4). In this case, however, we have reversed the sign of the electrokinetic potential from minus to plus, *i.e.*, we have passed the isoelectric point and the charge on the gel has been reversed.

Throughout all of these changes, both in the magnitude and sign of the ζ-potential, we have assumed little or no change in the thermodynamic potential (ϵ_b). Probably some change does take place in the

potential (ϵ_b). The change which takes place, however, is according to equation (59) directly proportional to the logarithm of the quotient of the ion concentrations in the two phases.

The electrokinetic potential, on the other hand, does not vary directly with the differences in ion concentrations across the entire interfacial region (as measured by the usual physicochemical technic within the gel and the body of the liquid) but rather with the ion con-

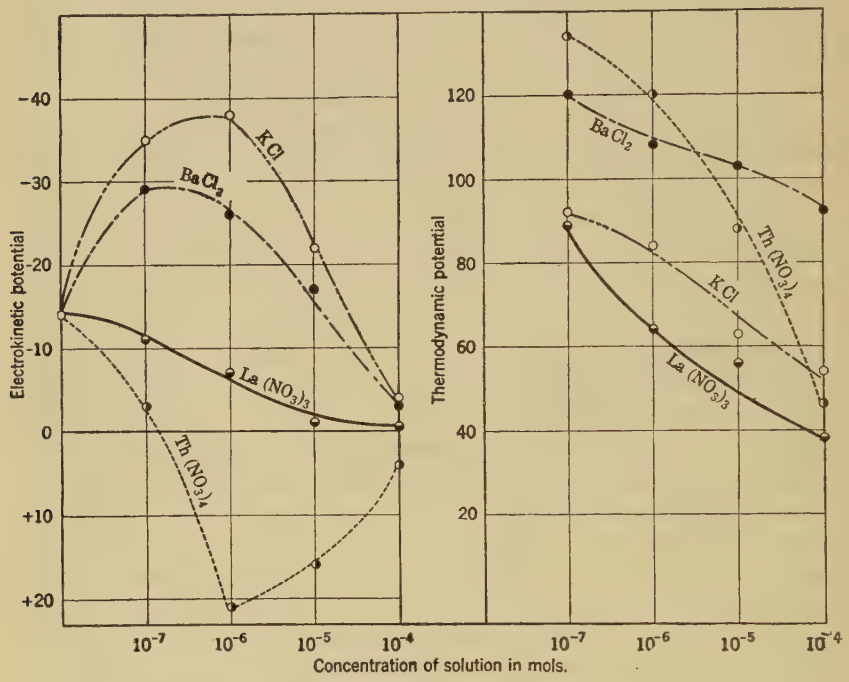


FIG. 45.—Showing that the ζ-potential and the thermodynamic or Nernst potential may vary independently of each other. (Data of Freundlich and Ettisch for a glass-water interface.) Note that change in the Nernst potential is approximately proportional to the concentration, whereas there is no predictable relation between the change in the electrokinetic potential and concentration.

centrations in the *thin film* which is adsorbed upon the micelles and the ion concentrations in the body of the liquid. In many instances the first unit addition of electrolyte to the body of the liquid may cause a very great change in the electrokinetic potential (probably due to the fact that either the anions or the cations of the added electrolyte are nearly all concentrated in the adsorbed immovable film), whereas a second unit addition of electrolyte to the body of the liquid may cause little or no change in (ζ). Figure 45, taken from the work of Freundlich and

Ettisch,[12] shows how the ζ-potential may vary independently of the thermodynamic potential.

It is admitted that Fig. 44 is a hypothetical diagram. It is believed, however, that this diagram and the assumptions which have been made afford a reasonable explanation for many of the reactions characteristic of colloid systems.

Loeb[13] based most of his argument that protein reactions were stoichiometrical reactions on studies involving the determination of the thermodynamic potential (ϵ_b) at phase boundaries between proteins and the liquid with which the protein micelles were in contact. It is not surprising that Loeb reached the conclusions that the boundary potential varied in a quantitative manner with the ion concentration of the solutions.

As Hill[14] points out, this conclusion was an inevitable result of the manner in which the determinations were made. We shall have occasion later to discuss certain of these problems. It is only necessary at this time to point out again that *whereas the electrokinetic potential is related to the thermodynamic potential, it may vary independently of that potential.*

Various methods have been proposed for the determination of the sign and magnitude of the electrical charge on colloidal micelles or surfaces. The methods which have been proposed may be classified as *cataphoresis, electro-endosmosis, streaming potential,* and *sedimentation potential.* All of these methods are interrelated and should be capable of yielding similar values.

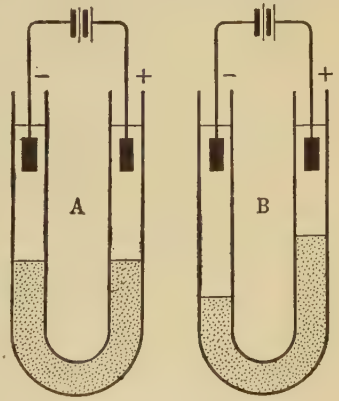


FIG. 46.—A diagrammatic representation of cataphoresis.

(*a*) CATAPHORESIS.—*Cataphoresis may be defined as the migration of the colloidal micelle through the dispersions medium, due to an imposed E.M.F.* Figure 46 shows diagrammatically a U-tube in which a colloid sol has been layered underneath a buffer solution having the same hydrogen ion concentration. Electrodes have been inserted in the buffer solution and connected to a source of direct current. Tube (*A*)

[12] Freundlich, H., and Ettisch, G., Das elektrokinetische und das thermodynamische Potential, *Z. physik. Chem.*, 116 : 401–419 (1925).

[13] Loeb, J., Proteins and the Theory of Colloidal Behavior, McGraw-Hill Book Company, Inc., New York (1922).

[14] Hill, A. V., The Potential Difference Occurring in a Donnan Equilibrium and the Theory of Colloidal Behavior, *Proc. Roy. Soc.*, (A), 102 : 705–710 (1923).

shows the position of the boundary between the sol and the buffer solution before any electric current has passed. Tube (*B*) shows diagrammatically the displacement of the boundaries, due to the passage of the electric current, on the assumption that the sol was negatively charged. It will be noted that the colloidal micelles have migrated toward the anode in the same manner as they would have migrated providing they behaved as ions. Various forms of apparatus have been devised for studying not only the direction of migration but also the rate of migration. The direction of migration indicates the sign of the charge on the colloid micelle, the colloid micelle migrating through the liquid toward the pole of the opposite sign. *The velocity of migration is proportional to the electrokinetic potential existing across the Helmholtz double layer.*

The equations which can be applied in cataphoresis studies are

$$v = \frac{\zeta E \epsilon}{4\pi\eta} \tag{60}$$

or

$$\zeta = \frac{4\pi v \eta}{E \epsilon} \tag{61}$$

where v = velocity of migration;
E = the applied E.M.F. per unit length between electrodes;
ϵ = the dielectric constant;
η = the viscosity.

Many papers have been published dealing with the technic of the measurement of the sign and magnitude of the electrical charge on suspended particles. It is practicable to note only a few of these papers in the present connection, the papers which have been selected being chosen either because of some novel form of apparatus which was used or because the papers present results which it will be desirable to refer to later in other connections.

Mattson,[15] Northrup,[16] Kunitz,[17] Northrup and Kunitz,[18] and Szent-Györgyi[19] have described apparatus suitable for measuring

[15] Mattson, S. E., Ein Mikroüberführungsapparat, *Koll. chem. Beih.*, 14 : 309–312 (1922).

[16] Northrup, J. H., The Stability of Bacterial Suspensions. I. A Convenient Cell for Microscopic Cataphoresis Experiments, *J. Gen. Physiol.*, 4 : 629–633 (1921–22).

[17] Kunitz, M., A Cell for the Measurement of Cataphoresis of Ultramicroscopic Particles, *J. Gen. Physiol.*, 6 : 413–416 (1923–24).

[18] Northrup, J. H., and Kunitz, M., An Improved Type of Microscopic Electrocataphoresis Cell, *J. Gen. Physiol.*, 7 : 729–730 (1924–25).

[19] Szent-Györgyi, A. v., Ein Kataphorese-Apparat fur kleine Substanzmengen, *Biochem. Z.*, 139 : 74–76 (1923).

cataphoretic velocity with small quantities of materials. The apparatus of Northrup, Kunitz, and Northrup and Kunitz is particularly adapted for studies involving microscopic objects, such as bacteria. Michaelis and Domboviceanu[20] have studied the cataphoresis of mastic sols. Kruyt and Arkel,[21] and Kruyt[22] outlined in detail precautions which, must be observed in measurements of cataphoresis. Svedberg and Tiselius,[23] and Scott and Svedberg[24] have studied the cataphoresis of egg albumin, using very novel technic. Figure 47 shows the cataphoresis tube which was used by Scott and Svedberg. The protein solution is inserted from reservoir (*C*) through stopcock (*B*) and is layered underneath a buffer solution, as shown in (*A*). (*D*) represents a connecting tube filled with buffer solution and dipping by means of a siphon into a saturated solution of zinc sulfate. The electrodes at (*E*) are zinc rods. By using a zinc-zinc sulfate electrode, polarization is prevented. Similarly non-polarizable electrodes of copper-copper chloride, and of silver-sodium chloride can be used,

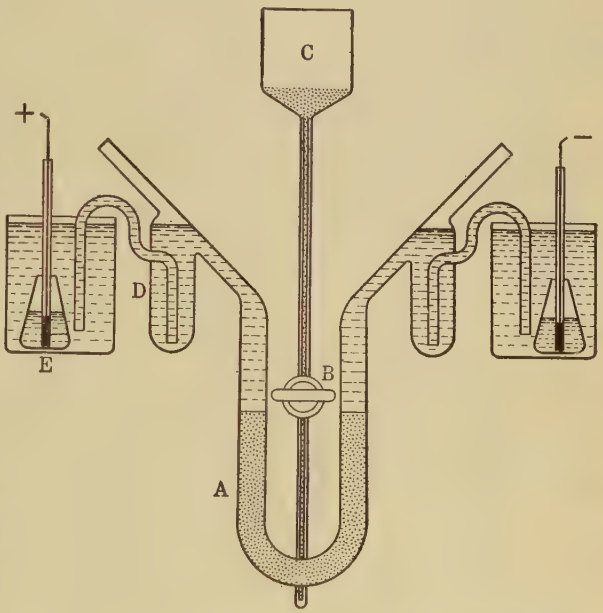


FIG. 47.—Svedberg's apparatus for conducting cataphoresis experiments.

[20] Michaelis, L., and Domboviceanu, A., Untersuchungen über die Kataphorese des Mastixsols, *Kolloid Z.*, 34 : 322–327 (1924).

[21] Kruyt, H. R., and van Arkel, A. E., Eine Methode zur ultramikroskopischen Messung der kataphoretischen Geschwindigkeit, *Kolloid Z.*, 32 : 91–95 (1923).

[22] Kruyt, H. R., Die Methoden zur Bestimmung der Ladungsgrösse kolloider Teilchen, *Kolloid Z.*, 37 : 358–365 (1925).

[23] Svedberg, The, and Tiselius, A., A New Method for Determination of the Mobility of Proteins, *J. Am. Chem. Soc.*, 48 : 2272–2278 (1926).

[24] Scott, N. D., and Svedberg, The, Measurements of the Mobility of Egg Albumin at Different Acidities, *J. Am. Chem. Soc.*, 46 : 2700–2707 (1924).

or one can use calomel electrodes dipping into a saturated solution of potassium chloride.

For exact quantitative work connection must be made through a reversible electrode; otherwise the buffer solution in the upper portion of the U tube, as shown in Fig. 46, changes in hydrogen ion concentration, and the diffusion of the acid or alkali formed by electrolysis might either coagulate the sol or alter the electrical charge, causing a corresponding change in velocity of migration.

Freundlich and Abramson[25] and Abramson[26] have likewise applied cataphoretic methods to the study of protein migration. Abramson's experiments will be referred to again.

(*b*) ELECTROENDOSMOSIS.—The theory of electroendosmosis has already been discussed in the early part of this chapter. *Electroendosmosis can be defined as the passage of a liquid through a membrane or colloidal gel under the force of an applied electric current.* The direction of migration of the water through the membrane or gel is toward the pole which has the same sign as is possessed by the membrane or gel. Figure 48 shows diagrammatically in (*A*) a membrane inserted in a tube, together with the liquid levels in the arms of the tube, before the electric current has been applied. In Fig. 48 (*B*) is represented diagrammatically the relative position of liquid in the two arms of the tube after an electric current has been applied to the electrodes, assuming that the gel was negatively charged. The direction of flow accordingly indicates the sign of the charge on the interface. *The volume of flow is proportional to the electrokinetic potential existing across the double layer.*

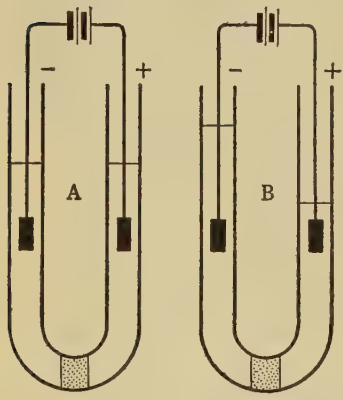


FIG. 48.—A diagrammatic representation of electroendosmosis.

The formulae which apply in electroendosmotic measurements are, for a single capillary tube,

$$v = \frac{r^2 \zeta H \epsilon}{4\eta l} \qquad (62)$$

[25] Freundlich, H., and Abramson, H. A., Über die kataphoretische Wanderungsgeschwindigkeit gröberer Teilchen in Solen und Gelen, *Z. physik. Chem.*, 128 : 25–38 (1927).

[26] Abramson, H. A., A New Method for the Study of Cataphoretic Protein Mobility, *J. Am. Chem. Soc.*, 50 : 390–393 (1928).

where (l) = the length of the capillary;
H = the E.M.F. applied across the diaphragm or between the electrodes, the other quantities being identical with those in equation (61),

and for a bundle of capillaries of cross section (q),

$$v = \frac{q\zeta H\epsilon}{4\pi\eta l} \tag{63}$$

and

$$\zeta = \frac{4\pi v\eta l}{qH\epsilon} \tag{64}$$

If the liquid is not allowed to flow out but accumulates so as to produce a hydrostatic pressure (P), the formulae then become,

$$P = \frac{2\zeta H\epsilon}{\pi r^2} \tag{65}$$

and

$$\zeta = \frac{P\pi r^2}{2H\epsilon} \tag{66}$$

Formulae (57) and (58) likewise apply to endosmotic flow.

Numerous papers have been published dealing with measurements involving electroendosmotic technic. Among the more interesting to the biochemist are those of Stamm[27,28] where sections of wood have been used as the membranes under investigation.

Most investigations on electroendosmosis have been conducted, using water as the liquid medium. However, Strickler and Mathews[29] have investigated the behavior of various organic liquids, using membranes of filter paper.

Briggs[30] has compiled an excellent review of electroendosmosis, together with its application to industrial processes. Some of the industrial applications suggest interesting possibilities. Thus, for example, it is pointed out that a considerable quantity of water can be removed

[27] Stamm, A. J., Electroendosmose through Wood Membranes, Colloid Symposium Monograph, Vol. IV, pp. 246–257, Chemical Catalog Company, Inc., New York (1926).

[28] Stamm, A. J., Effect of Electrolytes on Electroendosmose through Wood Membranes, Colloid Symposium Monograph, Vol. V, pp. 361–368, Chemical Catalog Company, Inc., New York (1928).

[29] Strickler, A., and Mathews H., Studies in Electric-Endosmose, *J. Am. Chem. Soc.*, 44 : 1647–1662 (1922).

[30] Briggs, T. R., Electrical Endosmose, Second Report on Colloid Chemistry, *Brit. Assoc. Advancement of Sci.*, pp. 26–52 (1918).

from colloidal gels by passing an electric current through the gel. For instance, peat can be compressed into blocks, electrodes applied at the two ends of the block, and when a current is turned on, water will flow from the moist peat to the cathode. Briggs points out that only water that is mechanically held in a gel can be removed by such a process.

In a similar manner, clay can be collected and partially dewatered. If one has a thin suspension of clay, the clay particles can be made to collect on the anode, due to cataphoretic migration, and later the mass of moist clay can be partially dewatered by subjecting the mass to conditions which will cause electroendosmotic flow. Briggs points out that Dawkins, in 1913, proposed a novel application of electroendosmosis to the process of brick manufacture. Wet clay has a marked tendency to adhere to a smooth metallic surface. In making wire-cut bricks, it is usual to employ a lubricant of some sort in order to prevent the clay from adhering to the cutting wires. It was found, however, that if the wires were connected with a source of direct current, the wire being made the cathode, the anode being inserted in the clay block, the clay would no longer stick to the wire, but that the wire would cut the clay cleanly, and with this electrical "lubrication" the power consumption was reduced by 25 to 30 per cent. Undoubtedly, as Briggs suggests, the action is due to water collecting in a film on the surface of the cutting wires due to electroendosmotic flow, this water film then acting as a lubricant for the wire, the mass of clay itself never coming in contact with the metal.

Crowther and Haines[31] have applied this method to a study of plowing. They point out that if the plowshare were made the cathode, being connected with an overhead power line, and the anode were imbedded in the soil of the field, it would be theoretically possible to lubricate the plowshare with a film of water so that the soil would never come in contact with the metal. They accordingly tested out this theory, using both laboratory-scale experiments and actual plowing tests.

A slider, consisting of a weighted steel slab, was so arranged as to be drawn by weights across the surface of a plane of moist soil. The weight necessary to keep the iron block in steady motion was considered to be a measure of the friction of the block upon the soil surface. Figure 49 shows the results which were obtained. In the absence of electrification of the slab a weight of 0.6 kilogram was necessary to keep the slab in motion. When the slab was made the anode, there was a sharp increase in the friction until at point (*B*) 1500 grams were necessary

[31] Crowther, E. M., and Haines, W. B., An Electrical Method for the Reduction of Draught in Ploughing, *J. Agr. Sci.*, 14 : 221–231 (1924).

to keep the slab in motion. At point (*B*) the current was shut off, the friction falling to point (*C*). The authors of the paper explain the higher friction at point (*C*) over that at point (*A*) as being due to the drying of the surface, the moisture being drawn into the interior of the soil. At point (*C*) the iron slider was made the cathode. Moisture, now, was drawn from the soil to the surface of the moving iron plate, thus forming a lubricating film between the iron and the soil, the weight necessary to move the iron plate dropping rapidly to point (*D*).

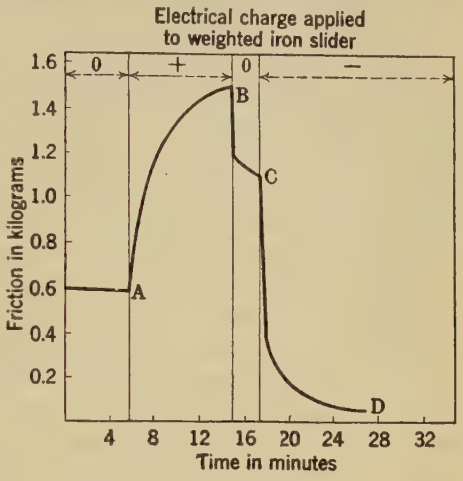


FIG. 49.—Showing how the friction between an iron slider and soil may be increased or decreased by electroendosmotic water flow.

Figure 50 shows a similar experiment in which the soil moisture content was varied and variations in electrical potential applied to the iron plate were introduced. It will be noted that the frictional force was reduced to approximately 20 per cent of its original value when soil containing 20 per cent moisture was used. When the soil contained only approximately 14 per cent of moisture, very little reduction in friction took place. Accordingly the moisture content of the material which is subjected to electroendosmosis becomes an important factor. It would appear that in the soil experimented with by Crowther and Haines, there was approximately 14 per cent of " bound water," *i.e.*, water adhering so firmly to the surface of the soil particles that it was no longer free to undergo endosmotic flow.

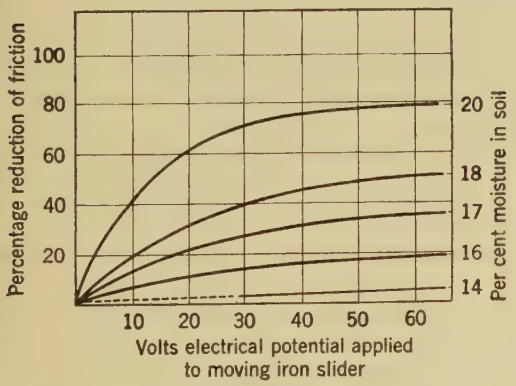


FIG. 50.—Showing the relationship between soil moisture and electroendosmotic water flow through the soil. (Data of Crowther and Haines.)

Crowther and Haines point out that in their actual plowing experi-

ments where the draw-bar pull was recorded by means of a dynamometer, there was a reduction in the energy necessary for plowing, but that the magnitude of the reduction was not sufficient to compensate for the current used.

(*c*) Streaming Potential.—It is obvious that if an electrical current induces a flow of liquid through a capillary, a condition which we have already discussed under electroendosmosis, the reverse phenomenon must take place, *i.e., forcing a liquid through a capillary will produce a difference in electrical potential between the two ends of the capillary.* The E.M.F. so induced is known as the *streaming potential.*

Kruyt,[32] Freundlich and Rona,[33] and Kruyt and Willigen[34] have developed technics for the quantitative estimation of the streaming potential and have developed formulae for the calculation of the ζ-potential from the streaming potential. The above mentioned workers have used a single glass capillary and have studied changes in the streaming potential produced by varying the kind of glass making up the walls of the capillary tube, as well as the effect of the various ions which are present in the liquid being streamed through the capillary. Kruyt found, using a glass capillary, that the electromotive force which was set up was directly proportional to the hydrostatic pressure and that the quantity, H/P, was a constant for a given capillary as long as the same solution was being forced through the capillary. Table XV, taken from Kruyt's paper illustrates this point.

TABLE XV

The E.M.F. Set Up between the Two Ends of a Glass Capillary when a Millimolar Solution of KCl Is Forced through the Capillary under Various Pressures.

P, Cm. Mercury	H, Millivolt	$\frac{H}{P}$
61.2	271	4.4
86.4	367	4.2
54.8	236	4.3
70.8	315	4.4

[32] Kruyt, H. R., Strömungspotentiale und Kolloidstabilität, *Koll. Z.*, 22 : 81–98 (1918).

[33] Freundlich, H., and Rona, P., Uber die Beziehungen zwischen dem elektrokinetischen Potentialsprung und der elektrischen Phasengrenzkraft, *Sitz. preuss. Akad. Wiss.*, 20 : 397–402 (1920).

[34] Kruyt, H. R., and van der Willigen, P. C., Strömungspotentiale und Kolloidstabilität, II. *Koll. Z.*, 45 : 307–319 (1928).

This table shows the magnitude of the potentials that may be expected.

Briggs[35] in an attempt to apply streaming potential methods to the determination of the electrokinetic charge on cellulose fibers observed that H/P was not a constant for different diaphragms of the same sample of cellulose. However, by introducing (κ_s), the specific electrical conductivity of the liquid as it exists inside of the diaphragm and which is being streamed through the diaphragm, into the streaming potential formula in place of (κ), the " bulk " specific conductivity of the liquid which was being streamed through the diaphragm, he was able to obtain a constant ratio of potential difference to the pressure used to induce streaming. Briggs accordingly proposes the formula,

$$\zeta = \frac{4\pi\eta\kappa_s H}{P\epsilon} \tag{67}$$

where η = the viscosity of the liquid being streamed through the diaphragm;
P = hydrostatic pressure under which the liquid flows;
ϵ = the dielectric constant of the liquid;
κ_s = the specific electrical conductivity of the system, *i.e.*, the liquid as it exists in the pores of the diaphragm material;
H = the potential difference existing across the diaphragm.

As Briggs notes, the values that must be determined are (P), (H), and (κ_s), and from these the electrokinetic potential can be calculated. The coefficient of viscosity for dilute aqueous solutions can be taken as 0.01. (ϵ), the dielectric constant of water, is considered as having a value of 80. (P), which is observed in centimeters of mercury, must be converted into dynes in order to express it in absolute units. Accordingly the height of the mercury column in centimeters must be multiplied by the specific gravity of mercury, 13.6, and the gravity constant, 981 dynes. (H), which is read in millivolts, must be divided by 1000 to reduce it to volts, and again divided by 299.86 to reduce volts to cgs electrostatic units. (κ_s) observed in reciprocal ohms must be multiplied by 9×10^{11} to convert it into cgs electrostatic units. The value of (ζ) so obtained would be in electrostatic units. In order to obtain this value in volts it must be multiplied by 299.86. Then

$$\zeta = \frac{H\kappa_s}{P} \times \frac{9 \times 10^{11} \times 4 \times 3.1416 \times .01 \times 299.86}{13.6 \times 981 \times 10^3 \times 299.86 \times 80}$$

$$= 1.0596 \times 10^2 \times \frac{H\kappa_s}{P}$$

[35] Briggs, D. R., *J. Phys. Chem.*, 32 : 641–675 (1928).

where (ζ) is expressed in volts, (H) in millivolts, (κ_s) in reciprocal ohms, and (P) in centimeters of mercury.

The apparatus which was developed by Briggs is shown in Fig. 51. (T) is a pressure tank of about 50 liters capacity into which air is pumped until the desired pressure is attained. This air passes through a washing bottle into reservoir (R), containing the liquid which is to be streamed through the diaphragm, the pressure on the liquid being measured by the mercury manometer. Stopcocks S_1 and S_2 are used to regulate the pressure on the manometer, stopcock S_3 being used to stop liquid flowing through the cell. The cell shown in the upper left-hand corner of the figure is a combination conductivity cell and streaming potential cell. Material to make up the diaphragm is packed into the center glass compartment (C). Two perforated gold electrodes (E) are pressed tightly against this diaphragm material and two glass end compartments with heavy glass flanges are then fitted against the electrodes, and the cell is clamped tightly together. Thin rubber washers can be placed between the center compartment (C) and the gold electrodes, and between the end compartments (G) and the gold electrodes, so as to make the cell water-tight. To the edge of each electrode is soldered a heavy platinum wire which will dip into mercury cups from which contact is made to the measuring devices. The potential which is set up between the two electrodes is measured by use of a quadrant electrometer. As soon as this reading has been made, the electrical conductivity of the system is taken by means of the usual Wheatstone bridge set-up, and later the cell constant of the cell containing the diaphragm material is determined by the usual technic for determining the cell constant, *i.e.*, by replacing all liquid in the cell and diaphragm with $N/10$ KCl, measuring the resistance of this system and calculating the cell constant in the usual manner.

Using the above set-up, Briggs was able to demonstrate differences in the electrokinetic potential on different samples of cellulose.

In a later paper[36] he showed that the electrokinetic potential on proteins could be accurately determined by preparing a quartz diaphragm and streaming through such diaphragm a very dilute solution of protein. In this manner protein was adsorbed upon the surface of the quartz powder, so that when equilibrium was reached, he no longer had a quartz surface but a protein surface supported on the quartz powder.

Using egg-albumin solutions of various hydrogen ion concentrations, he studied the effect of hydrogen ion concentration on the electrokinetic potential. The solid dots in Fig. 52 show the change in ζ-potential of

[36] Briggs, D. R., The Measurement of the Electrokinetic Potential on Proteins by the Streaming Potential Method, *J. Am. Chem. Soc.*, 50 : 2358–2363 (1928).

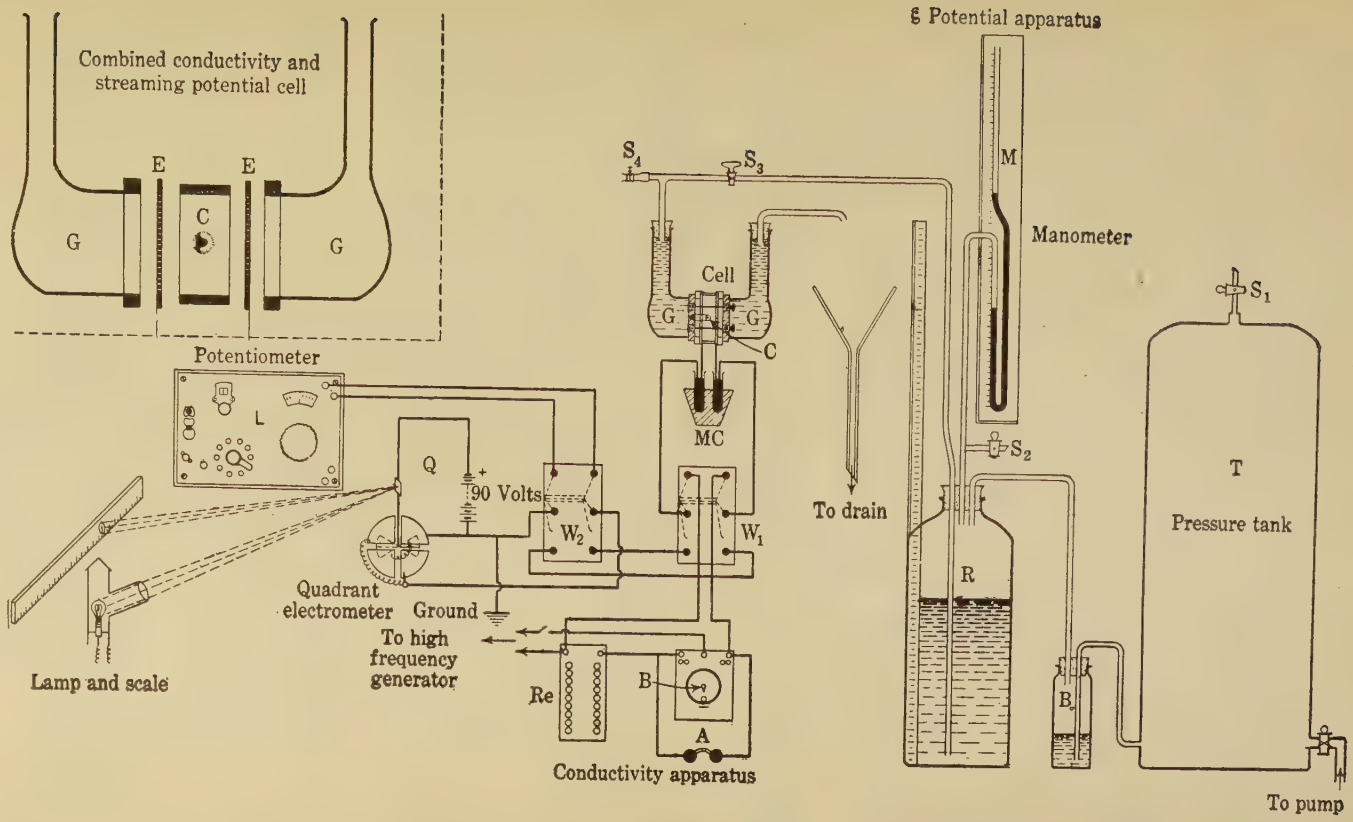


FIG. 51.—Diagram of apparatus for determination of the electrokinetic potential by the "streaming potential" method. (After Briggs.)

such an egg-albumin-quartz membrane at varying hydrogen ion concentrations. The open circles in the same figure show similar data obtained by Abramson, using cataphoretic measurements. The results of these two workers, using different methods, agree surprisingly well and apparently indicate that the fundamental theories underlying the calculation of the ζ-potential are sound.

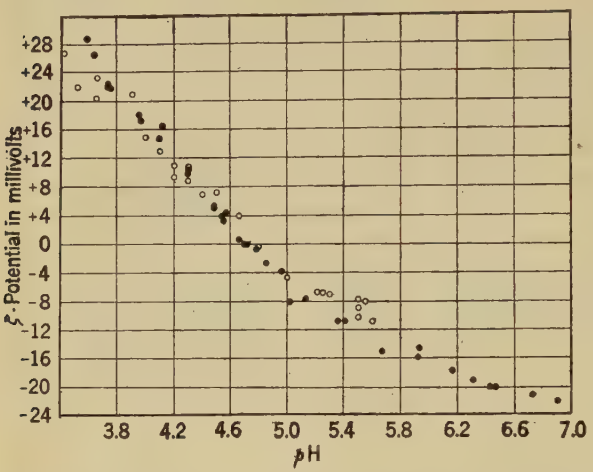


FIG. 52.—The variation of the ζ-potential with pH on egg albumin. Solid dots—by streaming potential method (Briggs). Circles — by cataphoresis, measurements by Abramson.

It will be noted that the ζ-potential of egg albumin falls to zero at approximately pH 4.7. Sörensen,[37] as the result of an elaborate series of experiments, found the isoelectric point of egg albumin to be at a hydrogen ion concentration of approximately 15.74×10^{-6}, which corresponds to a pH of approximately 4.80. The curves, therefore, agree well with Sörensen's figures. It will be noted that on the acid side of the isoelectric point the egg-albumin micelles possess a positive charge, whereas when the hydrogen ion concentration is less than that represented by pH 4.7, the micelles possess a negative charge. In Fig. 52 the four black dots nearest to the point of zero ζ-potential were obtained in three individual experiments, using a new membrane of egg albumin supported on quartz powder for each experiment.

Using streaming potential methods, Briggs[38] has likewise studied the influence of various ions on the electrokinetic potential. The results of this study will be referred to later.

(*d*) SEDIMENTATION POTENTIAL.—Dorn,[39, 40] in 1878, discovered

[37] Sörensen, S. P. L., Studies on Proteins. II. On the Capacity of Egg-Albumin to Combine with Acids or Bases, *Compt. rend. Lab. Carlsberg*, 12 : 68–163 (1917).

[38] Briggs, D. R., The ζ-Potential and the Lyotropic Series, *J. Phys. Chem.* 32: 1646–1662 (1928).

[39] Dorn, E., Ueber die galvanischen Ströme, welche beim Strömen von Flüssigkeiten durch Röhren erzeugt werden, *Ann. d. phys.*, 5 : 20–44 (1878).

that when particles fall through a liquid, there is a difference of electrical potential established between the top and the bottom of the liquid. This is the converse of cataphoresis in the same way that the streaming potential is the converse of electroendosmosis. In cataphoresis the particles are drawn through the liquid under the influence of an electric

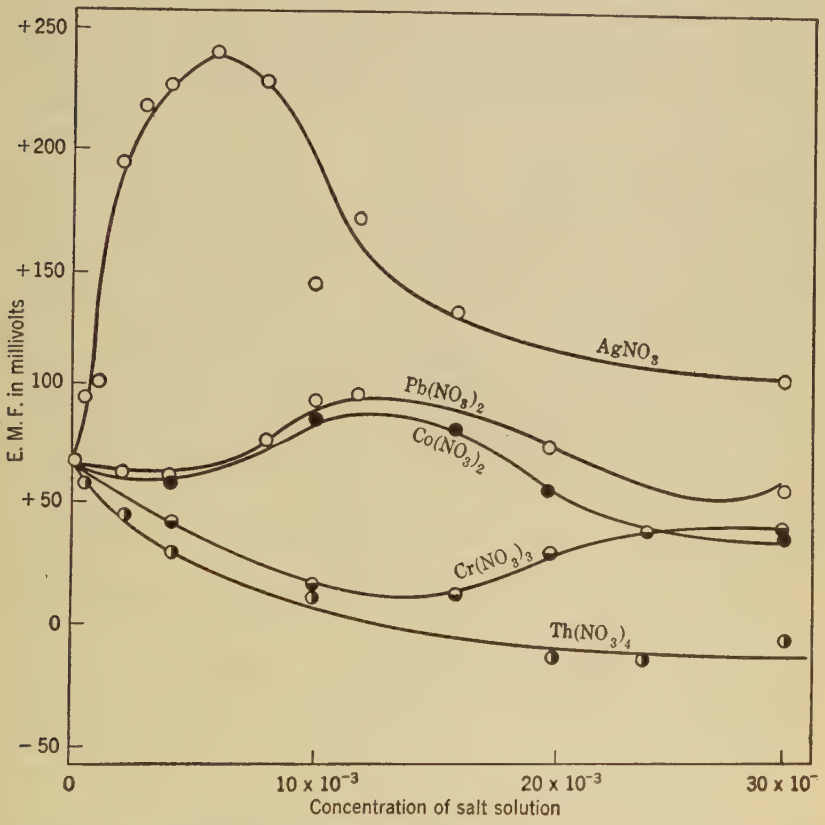


FIG. 53.—Showing the magnitude of and changes in the "sedimentation potential" of galena particles in various solutions. (Data of Bull.)

current. Accordingly *an E.M.F. is set up when particles are allowed to fall through a liquid.* Similar observations were made by Billitzer,[41]

[40] Dorn, E., Ueber die Fortführung der Electricität durch Strömendes Wasser in Röhren und verwandte Erscheinungen, *Ann. d. phys.*, 9 : 513–556; 10 : 46–76 (1880).

[41] Billitzer, J., Über die Elektrizitätserregung durch die Bewegung fester Körper in Flüssigkeiten, *Ann. d. Physik* (4) 11 : 937–956 (1903).

Freundlich and Mäkelt,[42] and Stock.[43,44] Stock allowed fine quartz powder to fall through nitrobenzene, ether, and toluene, and measured the magnitudes of the E.M.F. which was produced.

Bull[45] has recently applied this technic to the determination of the sign of the electrical charge on lead sulfide, and has attempted to explain the action of certain toxic ions in the industrial process known as ore flotation. While Bull did not attempt to determine the absolute value of the electrokinetic charge on the galena particles, he was nevertheless able to show that galena possesses a positive charge when in contact with pure water and that certain ions may greatly increase the magnitude of the charge, whereas other ions cause a pronounced decrease in the magnitude of the charge.

Bull allowed crystalline galena, which had been ground to pass a 100-mesh sieve and which was retained on a 325-mesh sieve, to fall through a glass tube 65 centimeters in length and 3 centimeters in diameter, containing either water or dilute solutions of certain salts. Platinum wires, fused into the walls of the glass tube 2 centimeters from each end, were used as electrodes, and a quadrant electrometer was used to determine the sedimentation potential induced by the falling galena. Figure 53 shows the results which were obtained. In this figure the observed E.M.F. in millivolts is plotted against the concentration of the salt solution which was used.

[42] Freundlich, H., and Mäkelt, E., Über den absoluten Nullpunkt des Potentials, *Z. für Elektrochem.*, 15 : 161–163 (1909).

[43] Stock, J., Über die durch Bewegung fester Körper in Flüssigkeiten hervorgerufenen electroosmotischen Potentialdifferenzen, *Anzeiger d. Akad. d. Wiss. Krakau* [A.], (1913) 131–144.

[44] Stock, J., Beiträge zur Kenntnis der elektrischen Endosmose, *Anzeiger d. Akad. d. Wiss. Krakau* [A.], (1914) 95–106.

[45] Bull, H. B., The Electrostatics of Flotation, *J. Phys. Chem.*, 33 : 656–658 (1929).

CHAPTER VI

SURFACE TENSION, INTERFACIAL TENSION, SURFACE ENERGY, AND ADSORPTION

We have already discussed under emulsions certain phenomena which are dependent upon surface tension or interfacial tension changes. We have noted that an efficient emulsifying agent is one which lowers the interfacial tension between the two mutually insoluble phases. In order, however, to discuss adequately certain fundamental properties of colloid systems, it is necessary to consider in somewhat more detail the forces which operate to produce the phenomena which are designated as surface tension and interfacial tension. Such a discussion will necessarily be limited to the barest outline which will be adequate to lay the foundation for the discussions which are to follow.

Willows and Hatschek [1] have presented a most excellent discussion of surface tension and surface energy and their influence on chemical phenomena. The reader is referred to their book for a more complete elaboration of the theory. Likewise, Rideal [2] has devoted a considerable part of his book to these questions.

Surface Tension.—The energy of a gas is due solely to the kinetic energy of the gas molecules. Due to kinetic energy, the gas molecules are in constant motion, and an incessant bombardment of an individual gas molecule by the surrounding molecules takes place. As the gas molecules come closer together, due to compression, the chances of collision are increased per unit of mass. Consequently the energy of a gas is increased as the volume decreases and is diminished as the volume increases, the mass being kept constant. Accordingly as we allow a gas to expand so as to increase its volume, the net energy of the system is decreased. Conversely, compressing a gas so as to decrease its volume causes an increase in the energy of the system.

A liquid differs from a gas in that its molecules are closer together and the liquid is capable of assuming a definite form, *i.e.*, it has a boundary

[1] Willows, R. S., and Hatschek, E., Surface Tension and Surface Energy, Third Edition, J. and A. Churchill, London (1923).

[2] Rideal, E. K., An Introduction to Surface Chemistry, University Press, Cambridge, England (1926).

surface. A gas always completely fills the container in which it is placed, no matter how large the container may be. It is in this respect that it differs from a liquid which occupies a more or less fixed volume. A liquid possesses kinetic energy similar to a gas, but due to the fact that it has a surface, it likewise possesses surface energy, and it is this surface energy which confers upon liquids many of the properties which are not possessed by gases.

The surface of a liquid differs from the body of the liquid, in that the molecules making up the surface are largely oriented in some particular direction. The molecules in the body of the liquid are apparently distributed at random, but the work of Hardy, Harkins, Langmuir, Adam and others, has shown that the molecules in the surface film of liquids are in general arranged in an orderly fashion. This question of molecule orientation will shortly be discussed at length. Suffice it to say here that the surface of a liquid behaves as if there were a " skin " drawn over the bulk of the liquid, differing in physical properties and in molecular arrangement from the bulk of the liquid beneath the surface layer. The concentration of molecules per unit area in this surface layer is usually greater than it is in an equivalent volume within the bulk of the liquid. The layer of molecules on the surface is more or less rigid and gives rise to the phenomenon which we call surface tension.

Surface tension is due to molecular cohesion. According to LaPlace, molecules in a liquid have a pronounced attraction for each other. This attractive force, however, operates over only a short distance and is greatest at not to exceed one or two molecular diameters. It is probably negligible at a distance of 5 millimicrons but is quite large as we approach one molecular diameter. Figure 54 shows diagrammatically the force which must be overcome in bringing a molecule from the interior of the liquid phase into the surface of the liquid and from the surface of the liquid into the vapor phase. We have at (*A*) within the body of the liquid a molecule which is attracted equally from all sides by other molecules. However, as this molecule approaches and touches the surface at (*B*), it is held back from escaping into the vapor phase by the molecular cohesion with the surrounding molecules. The arrows in the

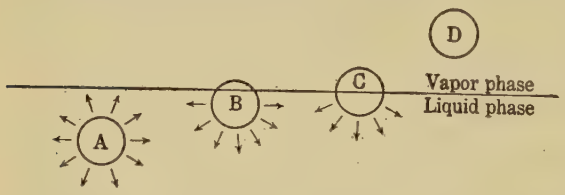


FIG. 54.—A diagrammatic representation of the direction and degree of the forces of molecular attraction acting upon a molecule, (*A*) in the body of the liquid, (*B*) touching the surface, (*C*) in the interface, and (*D*) in the vapor phase.

diagram show the direction of this cohesional attraction. It will be noted that at (*A*) there is an equal pull in all directions; at (*B*) there is an excess of downward and lateral pull. In order for the molecule to reach the interfacial film at (*C*), energy must be expended to overcome a part of the downward and lateral attractions, and a further amount of energy must be expended to overcome the downward pull when the molecule passes from (*C*) into the vapor phase at (*D*). This downward and lateral pull of molecules which are in the surface film or which are entering the surface film is the tension which we measure and call surface tension. An excellent example to show surface tension is diagrammatically represented in Fig. 55. A wire loop (*ABCD*) is constructed and a movable cross-bar (*EF*) is placed upon this loop. The area (*BCEF*) is then covered with a liquid film. For experimental demonstration a soap solution film may be used. The cross-bar (*EF*), sliding on the wire loop under the force of gravity, will reach an equilibrium position at a given distance from (*BC*). If in this equilibrium condition additional weights are added at (*G*) to the cross-bar, the film (*BCEF*) will be stretched a certain distance until a new equilibrium is reached. An additional weight added at (*G*) will cause a further increase in the area of the film to a new equilibrium, and this can be continued until the film breaks. If the area (*BCEF*) of the film is known and we know the weight which is applied at (*G*), we can calculate the pull due to molecular cohesion on a unit surface of the film. We have here two opposing forces, molecular cohesion which causes the film to remain intact, and the opposing weight at (*G*), tending to pull the molecules apart.

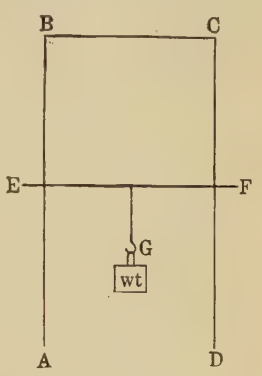


FIG. 55.—A diagrammatic representation of the fact that work must be done to increase surface area.

In the film in question we are dealing with two surfaces, one for each side of the film. The work done, expressed in ergs, to increase a surface by one square centimeter is numerically equal to the surface tension. Surface tension is usually expressed in ergs per square centimeter, where an erg is the work done when one dyne moves the point to which it is applied through a distance of one centimeter parallel to the direction of the force. The symbol (γ) has been chosen to indicate surface tension or interfacial tension,[3] although in the older literature (σ) is generally employed.

[3] International Critical Tables, Vol. I, p. 17, McGraw-Hill Book Company, New York, (1926).

TABLE XVI

Showing the Surface Tension (γ) in cgs Units for Certain Liquid-Air Interfaces

	Temperature, ° C.	γ		Temperature, ° C.	γ
Chloroform	25	26.2	Glycerine	20	63.4
Methyl alcohol	20	22.61	Mercury	0	480.3
Ethyl alcohol	20	22.27	Hydrogen peroxide		
Ethyl ether	20	17.10	(56.06%)	0	77.46
Acetic acid	20	27.63	Water	0	75.64±0.1
Benzene	20	28.86		10	74.22±0.05
Benzaldehyde	20	40.04		20	72.75±0.05
Aniline	20	42.58		25	71.97±0.05
				30	71.18±0.05

Table XVI shows the surface tension of certain common liquids. *Surface tension decreases with an increase in temperature and becomes zero at the critical temperature.* The decrease in surface tension is directly proportional to the temperature except possibly for a short range close to the critical temperature. Accordingly if we know the surface tension of a given liquid at two different temperatures, we can then draw a line between these points on coordinate paper, extrapolate the line, and determine the surface tension at other temperatures.

Referring again to Fig. 54, we have noted that energy is expended in bringing molecules into the surface film and in removing a molecule from the surface film into the vapor phase.

Stefan,[4] in 1886, proposed a generalization known as Stefan's law, in which he stated that one half of the latent heat of vaporization of a liquid was expended in pulling a molecule of the liquid into the surface film, the remainder being expended in pulling it out of the surface film into the vapor phase.

For example, the following processes are necessary to vaporize a molecule of water:

1. The water molecule must be brought from the body of the liquid to the surface layer.

2. The molecule must be pushed into and become a part of the surface layer of molecules.

[4] Stefan, J., Ueber die Beziehung zwischen den Theorien der Capillarität und der Verdampfung, *Wied. Ann.*, 29 : 655–665 (1886).

3. In order to vaporize this molecule, it must be forced out of the surface layer of molecules into the vapor phase.

The latent heat of vaporization of water is 540 calories at 100° C. Five hundred calories are expended in overcoming the pull of the neighboring molecules of liquid water. Only 40 calories are used to overcome the vapor density, atmospheric pressure, etc., *i.e.*, 40 calories represent the work done against the vapor phase, the remainder of the heat being utilized to overcome the downward pull of the liquid phase. If Stefan's law were strictly correct, we would have for water one half of the quantity, 21×10^9 dynes per gram of water, as the force necessary to bring the molecules of one gram of water into the surface film. Since 10^6 dynes is equivalent to 1 atmosphere pressure, we would have $\frac{0.5(21 \times 10^9)}{10^6}$, or 10,500 atmospheres pressure as the cohesional pressure of 1 gram of water.

Harkins and Roberts[5] have pointed out that *Stefan's law is only a rough approximation*, and that associated liquids and non-associated liquids differ markedly in their surface energy relations. Table XVII shows certain of Harkins and Roberts' data. In this table it will be noted that instead of half of the energy of vaporization being expended in pulling the molecule out of the surface film into the vapor phase as required by Stefan's generalization, the per cent energy expenditure for this process ranges from 55.71 per cent for liquid nitrogen to 84.91 per cent for water. Accordingly the calculated energy necessary to bring 1 gram of water into the surface film, as noted above, is in error.

Harkins and Roberts give the energy values for the vaporization of a molecule of water at 10° C. The total energy necessary for evaporation was found to be 69.6×10^{-14} ergs. Of this amount, 10.5×10^{-14} ergs was expended in drawing the molecule from the interior of the liquid into the surface film, and 59.1×10^{-14} ergs was expended in moving the molecule from the surface film into the vapor phase. It will be seen that we have here values which are widely divergent from the 50 : 50 ratio suggested by the generalization of Stefan.

Accordingly we can calculate the maximum surface area which can be occupied by one gram of water molecules at 10° C. Taking 0.37674×10^{10} ergs as representing the energy necessary to raise 1 gram of water from 10° C. to 100° C., and since 2.26×10^{10} ergs is the energy required to vaporize 1 gram of water at 100° C., we have 2.636×10^{10} ergs as the energy necessary to evaporate 1 gram of water at 10° C.

[5] Harkins, W. D., and Roberts, L. E., Vaporization in Steps as Related to Surface Formation, *J. Am. Chem. Soc.*, 44 : 653–670 (1922).

TABLE XVII

Showing for Some Common Liquids the Internal Latent Heat of Vaporization per Molecule (λ) and the Work Expended (e) in Bringing a Molecule from the Interior of a Liquid into the Surface Film and (j) in Causing the Molecule to "Jump" from the Surface Film into the Vapor Phase. The Energy Values are Expressed in 10^{-14} Ergs (Data of Harkins and Roberts).

Liquid	Absolute Temperature, Degrees	(λ)	(e)	(j)	$\left(\frac{e}{\lambda}\right)$ Per Cent	$\left(\frac{j}{\lambda}\right)$ Per Cent
Carbon tetrachloride	298	50.72	17.1	33.6	33.70	66.30
Ethyl ether	283.9	41.71	12.7	29.0	30.46	69.54
Benzene	298.1	52.8	19.5	33.3	36.93	63.07
Chlorbenzene	298	65.1	21.9	43.2	33.63	66.37
Ethyl acetate	303	53.1	16.0	37.1	30.13	69.87
Nitrogen	70	8.67	3.84	4.83	44.29	55.71
Oxygen	70	10.81	4.50	6.31	41.63	58.37
Methyl alcohol	413	43.2	10.1	33.1	23.38	76.62
Ethyl alcohol	383	55.6	11.7	43.8	21.04	78.77
Acetic acid	423	34.1	11.8	22.3	34.60	65.40
Water	283	69.6	10.5	59.1	15.09	84.91
Mercury	313	96.3	46.6	49.7	48.40	51.60

Of this amount, 15.09 per cent is expended in bringing the molecules from the interior of the liquid into the surface film. Accordingly, taking 71.94 ergs as the energy necessary to form one square centimeter of water surface at 10° C., we have 15.09 per cent of $\frac{2.636 \times 10^{10}}{71.94}$, or 55,292,000 square centimeters, as the area of surface film which can be covered at 10° C by 1 gram of water or a film $0.18 m\mu$ thick.

FIG. 56.—A diagrammatic representation of the direction and the intensity of the forces of adhesion acting upon a molecule in concave, convex, and plane surfaces.

The energy to remove a molecule from a surface film into the vapor phase is to some extent dependent upon the form of the surface. In Fig. 56 we have 3 molecules, molecule (D) in a concave surface, molecule (E) in a plane surface, and molecule (F) in a convex surface, the center of the molecule being spaced at an equal distance from the surface. It will be seen at a glance that more of the molecule protrudes

from the surface at (*F*) than at (*E*), and more at (*E*) than at (*D*). Accordingly there is the least cohesional downward and lateral pull of adjacent molecules on the moleçule at (*F*), and the greatest downward and lateral pull on the molecule at (*D*). The molecule at (*F*) accordingly can escape from the surface with less expenditure of energy than would be necessary for the molecule (*E*), and the molecule (*E*) can escape with less energy expenditure than can molecule (*D*). Accordingly molecules will spontaneously evaporate from surface (*F*) to surface (*E*), and from surface (*E*) to surface (*D*). This difference in surface energy relationships becomes important when we are dealing with very minute particles or droplets. The small droplets with a highly curved surface, as in (*F*), will spontaneously evaporate to larger droplets having more nearly plane surfaces. Correspondingly, a small particle having a highly curved surface will have a greater solubility in a given liquid than will a large crystal of the same material. As we shall see later, this is an important consideration when we are dealing with particles of colloidal dimensions.

Interfacial Tension.—Interfacial tension differs from surface tension only in that surface tension refers to a liquid-vapor interface, whereas interfacial tension refers to liquid-liquid or solid-liquid interfaces. No satisfactory method has yet been devised for measuring accurately interfacial tensions existing at solid-liquid interfaces. The interfacial tension of liquid-liquid interfaces can, however, be very conveniently measured by technic similar to that which is used for measuring the tension existing at liquid-vapor boundaries.

One of the earliest methods used for the measurement of surface tension involved the determination of the height to which a liquid would rise in a capillary tube inserted into a plane surface in the liquid. Diagram (*A*), Fig. 57, shows such a measurement. If we have a plane surface (*AB*) of a liquid in an open vessel, and into this plane surface a capillary tube is inserted and the liquid wets the wall of the capillary tube, the liquid will rise in the capillary, due to a difference in curvature of the surface within the capillary, since the vapor pressure at equilibrium with a concave liquid surface within the capillary is smaller than is the vapor pressure on the plane surface. Accordingly

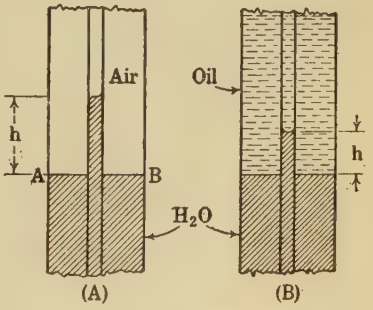


Fig. 57.—A diagrammatic representation of the relationship between surface tension and interfacial tension as measured by the capillary-tube method.

the liquid rises in the capillary to a height (h) which is equal to the difference in vapor pressure plus the weight of a column of liquid of height (h), which will compensate for the difference in vapor pressure of the liquid within the capillary. The surface tension of the liquid can accordingly be calculated from the formula:

$$\gamma' = \tfrac{1}{2}\, hrD \tag{68}$$

where γ' = surface tension in grams per square centimeter;
h = height, in centimeters, of the rise of the liquid in the capillary;
r = the radius of the capillary tube, in centimeters;
D = the specific gravity of the liquid in grams per cubic centimeter.

In order to convert (γ') into (γ), the surface tension, *i. e.*, into dynes or ergs per square centimeter, it is necessary to multiply by 980.1, the gravity constant.

Diagram (B), Fig. 57, indicates how a similar arrangement can be used to measure interfacial tension (cf. Pound[6]). The interfacial tension at the interface of two immiscible liquids can be calculated by the formula

$$\gamma = \frac{1}{2}\, Gr\lambda\mu \left(h + \frac{r}{3\mu}\right)(D - D') \tag{69}$$

where γ = interfacial tension in ergs per square centimeter;
G = 980.1 dynes;
r = radius of the capillary tube in centimeters;
λ = the ratio of the difference in level in the two liquids when the outer tube is of large diameter and the inner tube is of capillary size;
μ = the value of the scale division in centimeters;
h = difference in level of the two interfaces (the highest to the lowest points of the meniscus) inside and outside the capillary tube, in scale divisions;
D and D' = density of the two liquids, respectively.

Table XVIII shows certain values of the interfacial tension of pure liquids against a water surface, taken from the data of Pound. In a later paper, Pound[7] has determined the interfacial tension between 42 organic liquids and water or various aqueous solutions. He points

[6] Pound, J. R., Interfacial Tension, *J. Chem. Soc.*, London, 123 : 578–599 (1923).

[7] Pound, J. R., Interfacial Tensions between Organic Liquids and Water or Aqueous Solutions, *J. Phys. Chem.*, 30 : 791–817 (1926).

TABLE XVIII

Showing the Interfacial Tension Between Certain Pure Liquids and Water
(Data of Pound)

	Interfacial Tension, 30° C.	Interfacial Tension, 10° C.
Benzene	32.5	34.10
Ether	11.13	10.19
Chloroform	31.41	32.17
Carbon tetrachloride	42.75	
Carbon disulfide	46.31	
Toluene	34.6	
Aniline	6.00	
Nitrobenzene	24.1	
Turpentine	23.0	
Para aldehyde	9.6	
Amyl alcohol	4.89	
Amyl acetate	10.88	
Ethyl acetate	6.27	
Cresol	4.28	

out that the greater the mutual solubility is between the two liquids, the lower is the interfacial tension between them. He also notes that the interfacial tension between certain esters in contact with dilute acid or alkali solutions in water decreases with time, and ascribes this decrease to the hydrolysis of the esters and the formation of products of the hydrolysis which cause a decreased interfacial tension.

Interfacial tension can also be obtained from surface tension data using the theorem, that the interfacial tension is equal to the surface tension of substance (A) saturated with substance (B), minus the surface tension of substance (B) saturated with substance (A), or

$$\gamma_{AB} = \gamma_A - \gamma_B \tag{70}$$

Harkins and Humphrey,[8] Reynolds,[9] and Antonoff,[10-12] and Harkins and Zollman[13] likewise studied the question of the accurate measure-

[8] Harkins, W. D., and Humphrey, E. C., The Drop Weight Method for the Determination of Surface Tension, *J. Am. Chem. Soc.*, 38 : 228–241 (1916).

[9] Reynolds, W. C., On Interfacial Tension. I. The Statical Measurement of Interfacial Tension in Absolute Units. II. The Relation between Interfacial and Surface Tension in Sundry Organic Solvents in Contact with Aqueous Solutions, *J. Chem. Soc.* (London), 119 : 460–476 (1921).

[10] Antonoff, G., Über die Spannung an der Grenze zweier Flüssigkeitsschichten,

ment of interfacial tension and have presented data on various systems. Certain data of Harkins and Zollman have already been recorded in Table IV. The striking result of their work is the extremely low interfacial tension which was secured between olive oil and a 0.15 N solution of sodium chloride in 0.001 N sodium hydroxide. As already pointed out, a low interfacial tension between two mutually insoluble liquids provides ideal conditions for the formation of a permanent emulsion.

Various other methods for the measurement of surface tension and interfacial tension have been used. Thus, Morgan and Stevenson[14] showed that surface tension could be calculated from the weight of a drop falling from the tip of a capillary and that this technic gave more accurate values than the capillary-rise method. Harkins' values for interfacial tension were determined by a modification of this drop-weight method.

du Noüy [15] devised a torsion balance for measuring surface tension by recording the pull which is required to overcome the adherence of a ring of platinum wire to the surface of a liquid. This apparatus has recently been modified[16] so as to measure interfacial tensions. Green[17] has combined the torsion balance principle with the drop-weight principle and has devised an instrument to measure surface tension by recording the weight of a given number of drops of liquid. MacDougall[18] has pointed out certain limitations of the du Noüy tensiometer.

Undoubtedly changes in interfacial tension are responsible for many

J. Russian Phys. Chem. Soc., 39 : 342–353 (1907). Cited in *Chem. Zentr.* 1907, II, 1295.

[11] Antonow, G. N., Sur la tension superficielle à la limite de deux couches, *J. chim. phys.*, 5 : 372–385 (1907).

[12] Antonoff, G. N., Interfacial Tension and Complex Molecules, *Phil. Mag.*, (VI) 36 : 377–396 (1918).

[13] Harkins, W. D., and Zollman, H., Interfacial Tension and Emulsification. I. The Effects of Bases, Salts, and Acids upon the Interfacial Tension between Aqueous Sodium Oleate Solutions and Benzene. II. Extremely Small Interfacial Tensions Produced by Solutes, *J. Am. Chem. Soc.*, 48 : 69–80 (1926).

[14] Morgan, L. R., and Stevenson, R., The Weight of a Falling Drop and the Laws of Tate. The Determination of the Molecular Weights and Critical Temperatures of Liquids by the Aid of Drop Weights, *J. Am. Chem. Soc.*, 30 : 360–376 (1908).

[15] du Noüy, P. L., A New Apparatus for Measuring Surface Tension, *J. Gen. Physiol.*, 1 : 521–524 (1919).

[16] du Noüy, P. L., An Interfacial Tensiometer for Universal Use, *J. Gen. Physiol.*, 7 : 625–631 (1925).

[17] Green, R. G., The Surface Tension Balance. Apparatus for the Rapid Measurement of Surface Tension, *Ind. Eng. Chem.*, 15 : 1024–1025 (1923).

[18] MacDougall, F. H., Surface Tension Determined by the Ring Method, *Science*, 62 : 290–291 (1925).

of the phenomena which are characteristic of colloid systems. Emulsification appears to be primarily dependent upon interfacial tension changes. Interfacial tension changes are likewise involved in cell division. In 1876, Bütschli stated that if a drop of oil was suspended in an aqueous solution, the droplet would break into two drops providing that a high surface tension could be produced locally at the equator of the drop. Robertson[19] contended that the droplet would pull apart at the point where the surface tension was the lowest. McClendon[20] contended that Bütschli's view was correct and has carried out experiments designed to test the hypothesis. A chloroform-rancid olive oil droplet was suspended in a sodium chloride solution of specific gravity such that the oil droplet floated in the liquid. A dilute solution of sodium hydroxide was then allowed to flow from the tips of two pipettes against the opposite poles of the oil droplet. The alkali uniting with the acid in the droplet formed a soap which decreased the surface tension at the poles of the droplet and caused the droplet to elongate into an hour-glass form or to completely break in two, due to constriction by the higher interfacial tension at the equator.

Recently Bancroft and Gurchot[21] have repeated these experiments under somewhat more exact control and find that Bütschli was correct in his interpretation, as had already been indicated by McClendon's experiment. Undoubtedly interfacial tension is not the only factor operating in cell division. It does, however, provide a mechanical force which would be capable of producing the observed effects.

Surface Energy.—As already noted, a liquid differs from a gas in that it has a bounding surface. The energy of a gas is solely kinetic energy. The energy of a liquid, on the other hand, is composed of two factors, the internal or kinetic energy and the surface energy. The amount of kinetic energy available depends upon the initial value of the kinetic energy per unit of mass multiplied by the weight of the material. *The surface energy of a system is the product of two quantities, the intensity factor and the capacity factor.* The *intensity factor* is the *surface tension* or the *interfacial tension.* The *capacity factor* is the *extent of surface area.* The surface energy of a system can accordingly be expressed by the formula:

$$S = \gamma s \qquad (71)$$

[19] Robertson, T. B., Note on the Chemical Mechanics of Cell-Division, *Arch. Entwicklungsmechanik*, 27 : 29–34 (1909).

[20] McClendon, J. F., On the Dynamics of Cell Division. II. Changes in Permeability of Developing Eggs to Electrolytes, *Am. J. Physiol.*, 27 : 240–275 (1910).

[21] Bancroft, W. D., and Gurchot, C., Cell Mitosis, *J. Phys. Chem.*, 31 : 430–438 (1927).

where S = the surface energy;
γ = the surface tension or interfacial tension in ergs per square centimeter;
s = the surface area in square centimeters.

Surface energy is a very active form of energy and is readily converted either into work or into other forms of energy. Accordingly the free surface energy of a given system is decreased under many conditions. *A decrease in the free surface energy can be accomplished by* (*a*) *a reduction in area* (*reducing the capacity factor*) *or* (*b*) *a reduction of interfacial tension or surface tension* (*reducing the intensity factor*).

An example of the reduction of the surface energy by reducing the surface area is the coalescing of two mercury droplets into a single larger droplet. Such a coalescence takes place spontaneously when two clean mercury surfaces touch each other. The only way in which a pure liquid can decrease its surface energy is to decrease its surface area. Solutions, on the other hand, may have a higher or a lower surface tension than the pure solvent. In most instances, the surface tension of a solution is lower than that of the solvent, due to the fact that a solution may decrease its surface energy by bringing into the surface area an excess of the solvent or of the solute, depending upon which one lowers the surface tension to the greatest extent.

The surface energy of a pure solvent or of a crystalloidal solution is insignificant in comparison with the kinetic energy of the system, due to the fact that a relatively insignificant amount of surface is present. On the other hand, the surface energy in a colloid system may be relatively enormous, due to a very large absolute surface. The ratio of surface energy to kinetic energy is expressed by the term, specific surface, which is the ratio of the surface area of the disperse phase to the volume of the disperse phase,

$$\text{Specific surface} = \frac{\text{Absolute surface}}{\text{Volume}} \tag{72}$$

Table XIX shows the changes in total surface and specific surface of a hypothetical cube, one centimeter on an edge, which has been progressively subdivided until the particles have reached colloidal dimensions. It will be noted that the surface area has increased from 6 square centimeters to 60 square meters at the upper limit of the colloidal realm, and to 6000 square meters for a particle $1m\mu$ in diameter. The specific surface has increased from 6 to 60,000,000. During this great increase in specific surface, the internal energy or kinetic energy has remained constant. It is usually agreed that if the specific surface

TABLE XIX

Surface Area Possible by the Subdivision to Colloidal Dimensions of a Cube 1 cm. on an Edge

Edge of Cube	Number of Cubes	Total Surface	Relative Surface Energy,* Assuming That Water Is Being Subdivided, Ergs
1 cm.	1	6 sq. cm.	438
0.1μ	1,000,000,000,000,000	60 sq. m. (645.84 sq. ft.)	43,800,000
0.01μ	1,000,000,000,000,000,000	600 sq. m. (6458.4 sq. ft.)	438,000,000
$1m\mu$	1,000,000,000,000,000,000,000	6000 sq. m. (64,584 sq. ft.)	4,380,000,000 (105 cal.)

* The energy in this column is the calculated surface energy due to increased surface. No account has been taken of other possible forms of energy, such as electrical force, etc.

$\left(\frac{s}{V}\right)$ is less than 10,000, then the internal energy of a system predominates. On the other hand, if $\frac{s}{V}$ is greater than 10,000, then the surface energy becomes the predominating form of energy, and when the specific surface reaches such enormous values as are reached within the colloid realm, the reactions which take place are to a large measure reactions involving surface energy changes. It is for this reason that many substances which are totally different in their chemical composition, possess properties, when in the colloid state, which are similar or identical.

Every colloidal particle is surrounded by a film of the dispersions medium. Accordingly we have operating in these films an interfacial tension which controls, at least in a large measure, the energy relationships of a liquid-solid or a liquid-liquid interface in the same way that the surface tension expresses the energy relationships at a gas-liquid interface. While we can measure interfacial tension at a liquid-liquid interface, we are as yet unable to measure directly the forces of interfacial tension at a solid-liquid or a solid-gas interface. It is highly probable, however, that these forces are as great as, if not greater than, the known forces operating at liquid-liquid or liquid-gas interfaces. Even if we postulate forces no greater than those of liquid-gas interfaces, we have forces of sufficient magnitude to explain the surface reactions characteristic of colloid systems.

Wo. Ostwald[22] ascribes to Wenzel the law that "*the amount of chemical change in a unit of time is proportional to the absolute surface.*" Since colloid systems show a great absolute surface, they also show marked chemical activity.

Powdered sulfur is practically without action on a silver surface; colloidal sulfur, on the other hand, has an energetic action on a silver surface, causing the formation of the black silver sulfide. A smooth polished platinum sheet can be inserted into a solution of hydrogen peroxide without causing any appreciable amount of decomposition of the peroxide; if the platinum foil is roughened, a slow evolution of oxygen takes place; if powdered platinum is added to the hydrogen peroxide, the oxygen is formed rather rapidly; platinum black causes an energetic decomposition; and a colloidal platinum sol added to hydrogen peroxide may cause a violent explosion, due to the rapid evolution of oxygen. Colloidal platinum in a dilution as great as 1 gram atom of platinum in 70,000,000 liters of water can be detected by its decomposing action of hydrogen peroxide. No change in the chemical nature of the platinum has taken place, but the change in specific surface has shifted the chemical equilibrium in the same way that a rise in temperature would have shifted it. It is entirely probable that many of the reactions which we call catalytic reactions are in reality due to a change in the specific area of the substance used as the catalyst. In fact this may well be the predominating factor.

In a reaction taking place in a heterogeneous system, the tension at the interface is either increased or decreased according to the effect of the reacting product on interfacial tension. If the interfacial tension is increased, there is less energy available for the chemical reaction and the reaction slows up. If the interfacial tension is decreased, there is more energy available for the chemical reaction and the equilibrium is shifted toward an increased reaction. If salts of the fatty acids are brougbt into contact with a large surface area, increased hydrolysis takes place and an excess of fatty acids is concentrated in the interface with the excess of base in the liquid. This causes a disturbance in the equilibrium, shifting it to the right; consequently more of the fatty acid salt must be hydrolyzed to establish a new equilibrium:

$$MAc + H_2O \rightleftharpoons MOH + HAc$$

Powdering a substance before dissolving it, replaces work which must be expended in the solution process, by mechanical work. There is

[22] Ostwald, Wo., A Handbook of Colloid-Chemistry, translated by Martin H. Fischer, p. 93, P. Blakiston's Son and Company, Philadelphia (1915).

consequently more energy available for the work of solution of a small particle than of a large particle. Accordingly, finely powdered substances show a greater solubility and a greater heat of solution than do coarsely powdered substances or large crystals. Stas, in 1870, obtained the following values as the solubility of silver chloride: granular AgCl, 0.001 gm. per liter at 15°; powdered AgCl, 0.0060 gm. per liter at 17°; flocculent AgCl, 0.0140 gm. per liter at 20°.

Hulett[23-25] in a study of the solubility of gypsum found that particles 0.3μ in diameter had a solubility of 18.2 millimols per liter, whereas particles 2μ in diameter showed the normal solubility of 15.3 millimols per liter.

Hulett, similarly, found that small particles of barium sulfate of 0.1μ radius had a solubility of 4.15 millimols per liter, whereas particles of 1.8μ radius had the normal solubility of 2.29 millimols.

Hulett calculated from his data the interfacial tension of the solid-liquid. However, Freundlich[26] points out that the formula which Hulett used is incorrect. In its place, he proposes the formula,

$$\frac{RT}{M}\log_e\frac{C_2}{C_1} = \frac{2\gamma_{SL}}{rD} \tag{73}$$

or

$$\gamma_{SL} = \frac{RTDr}{2M}\log_e\frac{C_2}{C_1}$$

where R = the gas constant = 8.31×10^7 ergs;
T = the absolute temperature;
D = the density of the solid phase;
r = the radius of the smaller particles;
C_2 = the concentration of the solution in equilibrium with the particles of radius, r;
C_1 = the concentration of the solution in equilibrium with the larger particles, *i.e.*, the concentration of a solution in equilibrium with the massive solid phase;
γ_{SL} = the interfacial tension of the solid–liquid;
M = the molecular weight of the substance the solubility of which is being determined.

[23] Hulett, G. A., Beziehungen zwischen Oberfläschenspannung und Löslichkeit, *Z. physik. Chem.*, 37 : 385–406 (1901).

[24] Hulett, G. A., Löslichkeit und Korngrösse. Erwiderung an Herrn Prof. F. Kohlrausch, *Z. physik. Chem.*, 47 : 357–367 (1904).

[25] Hulett, G. A., Solubility and Size of Particles, Chapter 36, Colloid Chemistry, Vol. I, edited by J. Alexander, Chemical Catalog Company, Inc., New York (1926).

[26] Freundlich, H., Kapillarchemie, p. 144, Leipzig (1909).

Using this formula and Hulett's data, the interfacial tension of gypsum-water is found to be 1140 ergs per square centimeter and of the barium sulfate-water system, 1420 ergs per square centimeter.

Jones[27] later proposed a more complicated formula, taking into consideration the dissociation of the salts in the solution. His formula yields 1048 ergs per square centimeter for the gypsum system and 1332 ergs per square centimeter for the barium sulfate system.

Dundon and Mack,[28] and Dundon[29] have recently had occasion to repeat Hulett's experiments and extend them to other salts. They point out that Hulett's values for gypsum are too high, probably due to the fact that during the powdering of the gypsum a part of the crystal water was lost and that $CaSO_4 \cdot H_2O$ has a much greater solubility than gypsum.

TABLE XX

Surface Energy of Several Salts

Substance	Diameter of Particle, μ	Increase in Solubility, Per Cent	γ
PbI_2	0.4	2.0	130
$CaSO_4 \cdot 2H_2O$	0.5	4.8	356
$CaSO_4 \cdot 2H_2O$	0.2–0.3	12.3	385
Ag_2CrO_4	0.3	10.0	575
PbF_2	0.3	9.0	900
$SrSO_4$	0.25	26.0	1400
$BaSO_4$	0.1	80.0	1250

Table XX gives results obtained by Dundon and Mack, and Dundon. The last column in the table represents as nearly as can be determined with our present technic the probable interfacial tensions in ergs per square centimeter of the various substances against a water interface. Glasstone[30] found for lead oxide solubility values in normal sodium hydroxide solutions of 0.029 gm. mol. per liter for particles of 3

[27] Jones, W. J., Uber die Grösse der Oberfläschenergie fester Stoffe, *Z. physik. Chem.*, 82 : 448–456 (1913).

[28] Dundon, M. L., and Mack, E., Jr., The Solubility and Surface Energy of Calcium Sulfate, *J. Am. Chem. Soc.*, 45 : 2479–2485 (1923).

[29] Dundon, M. L., Surface Energy of Several Salts, *J. Am. Chem. Soc.*, 45 : 2658–2666 (1923).

[30] Glasstone, S., Physical Chemistry of the Oxides of Lead. Part I. The solubility of Lead Monoxide, *J. Chem. Soc.*, London, 119 : 1689–1697 (1921).

to 5μ in size, and a solubility of 0.040–0.049 gm. mol. per liter for particles of 0.7–1.5μ in size. From these values he calculated an interfacial energy between lead oxide and sodium hydroxide of 1860 ergs per square centimeter at 20°.

Lipsett, Johnson, and Maass[31] found a difference of 12.3 cal. per mole in the heat of solution of finely divided sodium chloride where the particles had an average diameter of 1.3μ, over that for the heat of solution of the coarsely crystalline salt. They point out that this 12.3 cal. is the heat which is bound up in the excess surface energy of the fine salt, and from their experimental values they find the surface energy of sodium chloride to range from 356 to 406 ergs per square centimeter.

We have already noted that colloidal particles may increase in size, due to crystal growth [cf. formula (3), p. 14] and that the rate of crystal growth depends in part upon the super-saturation of the solution. It must be obvious that, if small particles have a greater solubility than large particles and if a substance dispersed in small particles has a greater specific surface, and consequently a greater surface energy per unit of mass, small particles will dissolve in a solution which has not yet reached the saturation value for such particles but which is already super-saturated with respect to large crystals or a plane surface. Accordingly in a system containing particles of various sizes, equilibrium will eventually be established when all small particles have disappeared and only relatively large crystals are present.

Many papers have been written to present experimental work involving these considerations of crystal growth. Perhaps the most striking illustration is found in the work of Lipsett, Johnson, and Maass already referred to. They note that when their finely divided salt was placed in a moist atmosphere, water was rapidly absorbed, one gram of the salt absorbing 1 milligram of water in twenty minutes, 3 milligrams in two hours, and 6.9 milligrams in five hours. The 1 gram of salt originally occupied a volume of 8 cubic centimeters. At the end of five hours the volume had contracted to approximately one-fourth of its original bulk. A microscopical examination showed that whereas the original dry salt had contained innumerable particles approximately 1μ in diameter, after thirty minutes the main bulk of the salt was made up of particles ranging from 4μ to 10μ in diameter. They ascribe this change to the greater solubility of the small particles which dissolved in the water that was absorbed and then recrystallized as larger particles, and note that a few milligrams of water will act catalytically in transforming a large

[31] Lipsett, S. G., Johnson, F. M. G., and Maass, O., The Surface Energy and the Heat of Solution of Solid Sodium Chloride. I and II. *J. Am. Chem. Soc.*, 49 : 925–943; 1940–1949 (1927).

mass of finely divided salt into salt of much coarser degree of subdivision.

If finely divided materials possess an excess surface energy, they should melt at a lower temperature than larger particles. Here again, we have the principle of mechanical work expended in subdividing the particle, replacing thermal work. Palow[32, 33] has investigated the problem as to whether or not finely powdering a substance actually depresses the melting point. He finds that increasing the specific surface of salol (phenyl salicylate) one hundred times depresses the melting point 2.8° C. Accordingly we may find in this phenomenon an explanation as to why chemists do not always agree on the exact melting point of a chemical compound. If one chemist takes the melting point of relatively large crystals, and another chemist takes the melting point of very fine powder, we would expect the one using the large crystals to obtain the higher melting point.

This phenomenon may also explain in part why certain biological organisms are not frozen when the temperature falls to a few tenths of a degree below the freezing point, or why water in fine capillary spaces, such as the interstices of a clay soil, has a freezing point below 0° C. If water is finely divided, it should cause a depression of the temperature at which ice forms. Parker[34] has investigated the effect of mixing finely divided materials with water, benzene, and nitrobenzene. In each instance, he used materials which are insoluble in the liquid phase, so that the depression of the freezing point which was observed cannot be ascribed to the presence of a solution. It must be obvious from Table XXI that it is impossible to calculate the concentration of a solute in a solvent from freezing point data, if inert materials possessing large specific surface are present in the system at the time of making the freezing point measurements.

Reinders' Theorem.—Reinders[35] has investigated the distribution of a suspended powder or of a colloidally dispersed material between two mutually insoluble liquids. Let us assume, for example, that we have a red gold hydrosol, and this is mixed with petroleum ether. The problem is, will the disperse phase remain in the water or will it migrate to the petroleum ether, or will it form a layer at the interface between

[32] Palow, P., Über die Abhängigkeit des Schmelzpunktes von der Obenfläschenenergie eines festen Körpers, *Z. physik. Chem.*, 65 : 1–35; 545–548 (1908–9).

[33] Palow, P., Über die Schmelztemperatur der Körner des Salols, *Z. physik. Chem.*, 74 : 562–566 (1910).

[34] Parker, F. W., The Effect of Finely Divided Material on the Freezing Points of Water, Benzene, and Nitrobenzene, *J. Am. Chem. Soc.*, 43 : 1011–1018 (1921).

[35] Reinders, W. Von, Die Verteilung eines suspendierten Pulvers oder eines kolloid gelösten Stoffes zwischen zwei Lösungsmitteln, *Kolloid Z.*, 13 : 235–241 (1913).

the water and the petroleum ether. As Reinders has pointed out, the distribution will depend upon three interfacial tensions, the interfacial tension between solid and water (γ_{sw}), the interfacial tension between water and petroleum ether (γ_{wo}), and the interfacial tension between solid and petroleum ether (γ_{so}).

TABLE XXI

Change in the Freezing Point of Liquids When in the Form of Films on Finely Divided Powders (Data of Parker)

Substance	Liquid Added, Per Cent by Weight	Depression of Freezing Point Over That of Pure Liquid, ° C.	Substance	Liquid Added, Per Cent by Weight	Depression of Freezing Point Over That of Pure Liquid ° C.
	Water			Benzene	
Al_2O_3	25.0	2.118	SiO_2	3.3	0.670
Al_2O_3	30.0	1.227	SiO_2	5.0	0.490
Al_2O_3	35.0	0.650	SiO_2	10.0	0.225
Al_2O_3	40.0	0.370	SiO_2	20.0	0.110
	Benzene			Nitrobenzene	
Al_2O_3	30.0	1.337	Al_2O_3	50.0	1.720
Al_2O_3	35.0	0.682	Al_2O_3	60.0	1.175
Al_2O_3	40.0	0.492	Al_2O_3	70.0	0.810
Al_2O_3	50.0	0.212	Al_2O_3	80.0	0.580
			Al_2O_3	100.0	0.200

If $(\gamma_{so}) > (\gamma_{wo}) + (\gamma_{sw})$, the solid will remain suspended in the water.

If $(\gamma_{sw}) > (\gamma_{wo}) + (\gamma_{so})$, the solid will leave the water and go into the oil phase.

If $(\gamma_{wo}) > (\gamma_{sw}) + (\gamma_{so})$, or if none of the three interfacial tensions is greater than the sum of the other two, the solid particles will collect at the boundary between the water and the oil.

These conditions will also hold for a particle which comes in contact with a film of oil instead of a layer of oil or a globule of oil, and will determine whether or not the particle is wetted by the oil film. It will be wetted, if (γ_{sw}) > the other two interfacial tensions. It will remain in contact with the oil film, if (γ_{wo}) > the other two interfacial tensions. It will not be wetted, if (γ_{so}) > the other two interfacial tensions. These principles apply to practical problems and are particularly important in dealing with the adherence of insecticidal and fungicidal dusts and oil sprays.

Bartell and Osterhof[36, 37] have devised a new technic for measuring the relative "wettability" of various solids by different liquids, and from such studies one can determine which of the three conditions noted above is operating in a given system. Their technic allows one to determine not only which interface has the higher interfacial tension but also allows for a quantitative estimation of the intensity of the interfacial tension between solid and liquid. Studies, such as those of Bartell and Osterhof, are greatly needed in colloid chemistry, inasmuch as they afford quantitative measurements in systems where we have in the past been content with qualitative values.

The Orientation of Molecules in Surface or Interfacial Films.—We have already referred casually to the work of Hardy, Harkins, Langmuir, and Adam, as having shown that while the molecules in the body of a liquid are apparently distributed at random, those molecules which enter into the surface film are in general arranged in a more or less orderly fashion. While undoubtedly the germ of the idea of molecular orientation originated with Hardy,[38, 39] nevertheless Harkins,[40-43] and Langmuir,[44] independently of each other, have

[36] Bartell, F. E., and Osterhof, H. J., The Measurement of Adhesion Tension Solid against Liquid, Colloid Symposium Monograph, Vol. V, pp. 113–134, Chemical Catalog Company, New York (1928).

[37] Bartell, F. E., and Osterhof, H. J., Determination of the Wettability of a Solid by a Liquid, *Ind. Eng. Chem.*, 19 : 1277–1280 (1927).

[38] Hardy, W. B., The Tension of Composite Fluid Surfaces and the Mechanical Stability of Films of Fluid, *Proc. Roy. Soc.*, (A) 86 : 610–635 (1912).

[39] Hardy, W. B., The Influence of Chemical Constitution upon Interfacial Tension, *Proc. Roy. Soc.*, (A), 88 : 303–313 (1913); The Tension of Composite Fluid Surfaces. II., *Proc. Roy. Soc.*, (A), 88 : 313–333 (1913).

[40] Harkins, W. D., Brown, F. E., and Davies, E. C. H., The Structure of the Surfaces of Liquids, and Solubility as Related to the Work Done by the Attraction of Two Liquid Surfaces as They Approach Each Other. V. Surface Tension, *J. Am. Chem. Soc.*, 39 : 354–364 (1917).

[41] Harkins, W. D., Davies, E. C. H., and Clark, G. L., The Orientation of Molecules in Surfaces of Liquids, the Energy Relations at Surfaces, Solubility, Adsorption, Emulsification, Molecular Association, and the Effect of Acids and Bases on Interfacial Tension. Surface Energy VI., *J. Am. Chem. Soc.*, 39 : 541–596 (1917).

[42] Harkins, W. D., Clark, G. L., and Roberts, L. E., The Orientation of Molecules in Surfaces, Surface Energy, Adsorption, and Surface Catalysis. V. The Adhesional Work between Organic Liquids and Water, *J. Am. Chem. Soc.*, 42 : 700–712 (1920).

[43] Harkins, W. D., and Cheng, Y. C., The Orientation of Molecules in Surfaces. VI. Cohesion, Adhesion, Tensile Strength, Tensile Energy, Negative Surface Energy, Interfacial Tension, and Molecular Attraction, *J. Am. Chem. Soc.*, 43 : 35–53 (1921). (Cf. also Harkins' papers in Alexander's Colloid Chemistry, pp. 192–264, and Colloid Symposium Monograph, Vol. II, pp. 141–173.)

[44] Langmuir, I., The Constitution and Fundamental Properties of Solids and

developed this idea along somewhat different lines. The work of Harkins originated from his interest in the subject of energy at interfaces between liquids, while that of Langmuir originated from his work on liquid films. Both have come to the general conclusion that surface tension or interfacial tension phenomena are, in general, characterized by the orientation and the packing of molecules in surface layers and that the forces involved in this action are the forces of solution and the forces of interfacial tension.

Hardy[45] has extended his work in a different direction in studying the problems of friction and lubrication.

Concepts introduced by these workers may be summarized as follows. Liquids may be divided, in general, into two great classes, those in which the molecule is essentially symmetrical and those in which the molecule is more or less unsymmetrical. Pentane may be taken as an example of a symmetrical molecule, and acetic acid as an example of an unsymmetrical molecule. In the pentane molecule the two ends of the hydrocarbon chain are identical. Accordingly we would expect the two ends to behave identically toward an interface. In the case of acetic acid, on the other hand, the two ends of the molecule are very dissimilar, one being of hydrocarbon nature, the other (—OH) very similar to water. Accordingly we would expect the two ends of the acetic acid molecule to behave differently toward an interface. To express these differences the term, "*polar group*," has been introduced, and the radicals, —OH, —COOH, —CHO, —CN, —$CONH_2$, —SH, —NH_2, —$NHCH_3$, —NCS, —COR, —COOM, —COOR, —NO_2, —$CH{=}CH_2$, —$C{\equiv}CH$, and groups which contain oxygen, nitrogen, sulfur, iodine, bromine, and chlorine, and double- and triple-bonds have been called polar groups, and compounds containing these groups have been designated as *polar compounds*.

In the future discussion of molecular orientation, we will limit ourselves more or less to a consideration of a water-organic liquid or organic liquid-air interface.

It will be noted that a polar group confers upon an organic compound a certain solubility in water. Thus, for example, we have in methane a gas which is relatively non-water-soluble. When we introduce a polar group, as in methyl alcohol, methyl amine, etc., we form a compound which is water-soluble. One portion (CH_3—) of a molecule

Liquids. II. Liquids, *J. Am. Chem. Soc.*, 39 : 1848–1906 (1917). (Cf. also Colloid Symposium Monograph, Vol. III, pp. 48–75.)

[45] Hardy, W. B., Friction, Surface Energy and Lubrication, Chap. XIII of Colloid Chemistry, Theoretical and Applied, Vol. I, edited by Jerome Alexander, Chemical Catalog Company, Inc., New York (1926).

of methyl alcohol is of hydrocarbon nature, the other portion (—OH) is closely allied to water, and as such shows an affinity for water. If then a small amount of methyl alcohol is dissolved in water, it will concentrate in the surface film, due to the fact that methyl alcohol will lower the surface energy at an air-water interface. The fact that the (CH_3—) group retains a part of the properties of a hydrocarbon, and the (—OH) group retains a part of the properties of water causes the methyl alcohol molecules to orient themselves in the interfacial film, with the hydrocarbon chain toward the vapor phase and the (—OH) group toward the water phase. As we lengthen the hydrocarbon chain in passing to ethyl alcohol, propyl alcohol, butyl alcohol, amyl alcohol, etc., there is a progressive intensification of the hydrocarbon properties residing in the molecule, and a corresponding lessening in the similarity of the molecule to a water molecule. Accordingly the solubility of the alcohols in water decreases as the carbon chain is lengthened, and correspondingly their solubility in organic solvents increases.

In the case of the acids, when we approach lauric acid, we reach a point where the interface between an aqueous solution of lauric acid and air is essentially a hydrocarbon interface. Figure 7 has already been referred to as showing the effect on surface tension of the sodium salts of the saturated fatty acids of carbon chains of varying lengths. The slopes of these curves and the effect of the compounds on surface tension become clear when the theories of molecular orientation and polar groups are understood.

Harkins likens a polar group to a metallic weight attached to logs of wood. If the metallic weight is kept constant and exceeds in specific gravity the weight of the log, the log will be immersed in water. This is what happens when methyl alcohol is dissolved in water. The affinity of the (—OH) group for water is so great as to overcome the effect of the hydrocarbon chain. If, however, the log of wood is increased in size or in length, we eventually reach a point where the weight no longer will submerge it and the wooden end of the log will project above the surface of the water, the weight at the bottom tending to hold the log in an erect position.

If we imagine a bar of liquid which we divide into two parts by an imaginary plane, when the imaginary plane is lifted, the upper layer rises with it and two surfaces appear where there was no surface previously. In these two surfaces we will have a rearrangement of the molecules of the liquid from a random distribution, such as is characteristic of the interior of liquids, to one where the molecules are oriented in a more or less orderly fashion. Figure 58 is a diagrammatic example of such surfaces.

Similarly, if we dissolve butyric acid in benzene and pour this solution upon a water surface, the water will have a greater affinity for the carboxyl group of the butyric acid than it will have for the hydrocarbon chain, whereas, on the other hand, benzene will have a greater affinity for the hydrocarbon chain than for the carboxyl group, and these two opposing forces will cause an orientation of the butyric acid at the benzene-water interface, similar to the orientation diagrammatically shown in Fig. 59.

Harkins and his coworkers have shown that polar liquids are, in general, mutually soluble and that slightly polar liquids, such as hexane or octane, are soluble in other slightly polar liquids but are relatively

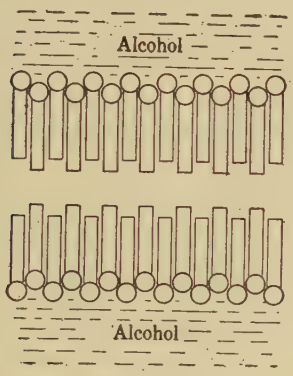


Fig. 58.

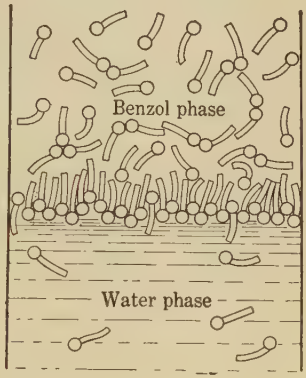


Fig. 59.

Fig. 58.—A diagrammatic representation of the orientation of molecules on surfaces when a column of alcohol is broken apart to give two surfaces. (After Harkins.)

Fig. 59.—A diagrammatic representation of butyric acid distributed between water, benzol, and the interface between the two. (After Harkins.)

insoluble in very polar liquids. It is the old theorem that " like attracts like." In general, the introduction of polar groups causes hydrocarbons to become soluble in water when the hydrocarbon chains are short. As the length of the hydrocarbon chain increases, there is a decrease in the free energy of a water interface up to a point where a hydrocarbon-water interface is formed, after which the free energy of the interface remains essentially constant.

As Langmuir has shown, the surface energy of the paraffin hydrocarbons from hexane to molten paraffin is essentially the same (46–48 ergs per sq. cm.), although the molecular weights differ very greatly.

From the above hypotheses, it is evident that the solubility of one substance in another may be the resultant of several opposing forces, or

in other words, that *different parts of a molecule may possess different affinities toward the solvent.* If one studies the behavior of various compounds containing different groupings, it is possible to predict with a fairly high degree of accuracy the solubility behavior of an unknown compound.

Harkins, Clark, and Roberts have measured the work which is required to pull apart a column of various organic liquids. This work is equal to twice the free surface energy of the liquid, inasmuch as two new surfaces are formed. They have termed this the " cohesional work " for a bar of unit cross section. Values are also given for the " adhesional work " against water. The adhesional work is the work done when one square centimeter of an organic liquid comes in contact with a similar area of water. This is a measure of the polar nature of the most polar part of the organic molecule. If $\frac{W_a}{2}$ is greater than $\frac{W_c}{2}$, then the work of attraction between the organic liquid and the water is greater than that between the parts of the organic liquid itself. Thus, the adhesional work is greater than the cohesional work. Consequently such substances should either dissolve more or less readily in water or should spread on a water surface. The actual magnitude of the values for the work of adhesion and the work of cohesion allows us to assign numerical values to indicate the degree of polarity of the various radicals.

When oleic acid is placed on water, it is probable that —COOH groups actually do dissolve in the water, *i.e.*, they are immersed in the water phase. The long hydrocarbon chains, however, have too much attraction for each other and too little for water to be drawn underneath the surface merely by the affinity of the —COOH group for water. Accordingly the oil spreads on the surface as a monomolecular layer, with the carboxyl groups immersed in the water. The spreading of an oil is thus due to the presence of a polar group in the molecule. If this be the case, an oil, such as paraffin oil, without a polar group should not spread on a water surface. Hardy found that many oils, such as the pure saturated aliphatic hydrocarbons, did not spread on a water surface, and explains this by stating that the great chemical stability of the paraffins makes chemical interaction with water impossible.

Harkins and McLaughlin,[46] using values derived from a study of adhesional and cohesional energies, have determined the number of molecules of butyric acid in a unit area of the interfacial film between water and hexane. The number of molecules which they found was

[46] Harkins, W. D., and McLaughlin, H. M., Monomolecular Films between Liquids: Butyric Acid between Water and Hexane, and Acetic Acid between Water and Benzene, *J. Am. Chem. Soc.*, 47 : 1610–1613 (1925).

2.78×10^{14} per square centimeter which corresponds to an area of 32 square Ångström units.[47]

Adam[48] has devised a method for measuring the area which can be covered by a given amount of material in a monomolecular film. Table XXII gives certain of his values for the surface area occupied by a single

TABLE XXII

Showing the Cross Sectional Dimensions of the Area Occupied by a Single Molecule in an Interfacial Film (Data of Adam)

Group	Cross Section, Å	Group	Cross Section, Å
Hydrocarbon chain	20.7	$-CH_2NH_2CONH_2$	25.5
$-CH_2CH_2COOH$	25.1	$-C_6H_4OH$	23.8
$-CH=CHCOOH$	28.7	$-C_6H_4NHCOCH_3$	28.2 or 25.8†
$-CH_2CH_2COOC_2H_5$*	22	Triglycerides	63
$-CH=CHCOOC_3H_5$	28.7	Glycol dipalmitate	42
$-CH_2OH$	21.7	Cholesterol	39
$-CONH_2$	<21	Hydrolecithin	53
$-CN$	27.5		

* Ethyl, methyl, and allyl esters pack into the same area.
† According to temperature.

molecule containing various groups. It will be noted that the first eleven substances in the table show some differences in cross-section area. Nevertheless they are relatively alike. When we pass, however, to a triglyceride, it is apparent that the glycerol portion of the molecule is lying flat in the interface with the three hydrocarbon radicals of the fatty acids projecting into the surface, inasmuch as the cross section of the triglyceride is approximately three times the cross section of the hydrocarbon chains. Similarly, in the dipalmitate we have two hydrocarbon chains projecting into the surface, with the glycol lying horizontally beneath.

In other papers, Harkins and McLaughlin,[49] and Harkins and Gilbert[50] have studied the structure of films on aqueous salt solutions.

[47] The Ångström unit is 1×10^{-8} cm.

[48] Adam, N. K., The Structure of Surface Films on Water, *J. Phys. Chem.*, 29 : 87–101 (1925).

[49] Harkins, W. D., and McLaughlin, H. M., The Structure of Films of Water on Salt Solutions. I. Surface Tension and Adsorption for Aqueous Solutions of Sodium Chloride, *J. Am. Chem. Soc.*, 47 : 2083–2089 (1925).

[50] Harkins, W. D., and Gilbert, E. C., The Structure of Films of Water on Salt Solutions. II. The Surface Tension of Calcium Chloride Solutions at 25°, *J. Am. Chem. Soc.*, 48 : 604–607 (1926).

They find the surface of a salt solution to be covered with a monomolecular water film which ranges in thickness from 4 Ångström units on a 0.1 M NaCl solution to 2.3 Ångström units on a 5.0 M NaCl solution. As we have already noted (Table XX), the surface tension of inorganic salts is relatively high; consequently an inorganic salt solution can decrease its free energy by bringing pure water molecules into the surface. Apparently, however, from these studies, the water film on the surface is monomolecular in thickness, the difference in cross section being explained by the intensity of molecular " packing."

From the above studies Harkins calculates that a water molecule occupies a volume equal to a cube 3.09×10^{-8} centimeters on an edge. He notes, however, that in these monomolecular films we are dealing not with a static film but with a film to which molecules are continually being added and from which molecules are continually escaping. He illustrates the kinetic energy of the surface by the following statement: "If a water surface at 20° is kept in a vacuum, 7,000,000 molecules jump out each second from the area occupied by a single molecule, so that at equilibrium an enormous number of molecules jump out from, and jump back into, this area. However, the time required for the orientation of a molecule of this type is so minute that at any instant the percentage of more or less oriented molecules is probably very high."

du Noüy[51, 52] from a study of surface tension has calculated the linear dimensions of the molecules of such materials as sodium oleate, egg albumin, serum albumin, etc., on the assumption that they form monomolecular layers. He noted that the materials studied could be divided into three groups. One group gave a single minimal value for the decrease of surface energy; another group gave two minimal values at different dilutions; a third group gave three minimal values. He explains this by assuming that in the first group the molecules had the form essentially of a cube, all three dimensions being approximately equal. Consequently, the area which would be covered by a unit weight of material in a monomolecular film, would be identical regardless of the direction in which the molecule was oriented.

For the second group he assumed two dimensions to be essentially equal with an unequal third dimension. A given quantity of such material could form a monomolecular film by covering the surface with the molecules packed in such a way that their greatest dimension lay in

[51] du Noüy, P. L., Surface Equilibria of Biological and Organic Colloids, Chemical Catalog Company, Inc., New York (1926).

[52] du Noüy, P. L., Some New Aspects of the Surface Tension of Colloidal Solutions Which Have Led to the Determination of Molecular Dimensions, Colloid Symposium Monograph, Vol. III, pp. 25–47 (1925).

the surface. However, on the addition of more material or by decreasing the surface area, the molecules would rearrange themselves to form a new monomolecular film with the square cross section in the surface and the greatest dimension of the molecule perpendicular to the surface.

The third group of substances he considered to have all three dimensions of the molecule unequal, thus allowing three monomolecular films to form, the first film with the molecules having their greatest dimension in the surface, on closer packing the intermediate dimension in the surface, and on closest packing the smallest diameter in the surface. The calculated dimensions for the molecules of sodium oleate were 6.64, 7.56, and 12.30 Ångström units, respectively. In crystalline egg albumin two of the dimensions were equal, the dimensions being 30.8, 30.8, and 41.7 Ångström units.

It has been possible to indicate only in barest outline some of the theories and applications involved in the problem of molecular orientation. It is hoped, however, that sufficient has been given to stimulate the interest of the student and direct him to literature sources, for in this field of molecular orientation lies undoubtedly an explanation for many biochemical and biological phenomena.

ADSORPTION.—As noted above, a solution may decrease its surface energy by bringing into the surface either an excess of the solvent or an excess of the solute, depending upon which one causes a lowering of the surface tension. In the same way a colloid system can decrease its surface energy by bringing into the interfacial film substances which lower the interfacial tension. *Those substances which decrease surface tension tend to concentrate in a liquid-vapor interface, and those substances which decrease interfacial tension tend to concentrate in a liquid-solid or a liquid-liquid interface. This phenomenon of concentrating at the interface is called* ADSORPTION.

Sodium oleate decreases the surface tension of water. If air is bubbled through a solution of sodium oleate, the foam which is carried over contains a higher percentage of sodium oleate than did the original sol, and the residual sol left behind is correspondingly weaker. A dilute acetic acid solution or a solution of certain dyestuffs may be used equally well to demonstrate the same phenomenon.

The phenomenon of adsorption, as a rule, does not bring into play the forces of primary valency, *i.e.*, it is not possible to write a stoichiometrical chemical equation to represent the process, for, after all, *adsorption is an equilibrium* depending upon the concentration of the material which is being adsorbed and the extent of surface upon which adsorption can take place. Mathews[53] has given a rather facetious and

[53] Mathews, A. P., Adsorption, *Physiol. Rev.*, 1 : 553–597 (1921).

at the same time graphic illustration of the adsorption process, as follows:

" To make clearer what is meant by adsorption, I may cite a typical case which is familiar to all and which is on so large a scale that the details of the process may be seen. Whenever we put on clothing, we adsorb the clothes. We and our environment are part of a great system. Any change of the environment produces a change in us, and any change in us produces some change in the environment. In this way we and the environment make what is known as a system, which consists of two constituents or phases, one of these being ourselves. As it has two phases or physically distinct parts, the system is heterogeneous. Every morning when we dress, clothing which has been distributed through our environment, dispersed in the surrounding phase in other words, concentrates at the surface of our bodies; or speaking technically, it concentrates at the interphase boundary of the system, so that the concentration of clothing at that boundary becomes greater than in the room about us. This is a process of positive adsorption. And at night the process is reversed, the concentration of clothing in the room becoming greater than at the surface of the body, the clothing passes out of the interphase, and we have then a case of negative adsorption. And we might go on, if we wished, with this treating of the wearing of clothes as a process of adsorption, express it mathematically in a curve or isotherm, showing how the quantity adsorbed is a function of the amount in the room; how it proceeds usually to an equilibrium; how it is greater at low than at high temperatures; that it is reversible and not accompanied by any chemical change in the clothes; that it is specific in that certain kind of clothes are adsorbed with greater avidity than others; that certain adsorbents (people) adsorb better than others; and finally we could prove that the clothing moved into the surface film in virtue of the second law of thermodynamics, and because of the thermodynamic potential in consonance with the principle of Willard Gibbs. In other words dressing is in every respect a typical case of adsorption, and if we wished to go farther we could actually prove, at least as well and to just the same degree as similar things are proved for protozoa and other living things so minute that we cannot easily see just what is happening, that the wearing of clothes is a surface tension phenomenon, and has a purely mechanical explanation. Human behavior, in the wearing of clothes, would thus be explained to the satisfaction of the mechanistic philosopher, and in the same manner as the wearing of mineral clothing, called shells, by protozoa has been explained. But the limits of this article are too short to permit of this demonstration, illuminating though it would be, and the case of dressing is here brought forward

simply to illustrate what is the essential characteristic of adsorption, namely a difference of concentration of some substance at the boundary of two phases, from its concentration in the phases."

Bayliss[54] has given a somewhat similar illustration as applying to enzyme action, on the assumption that the preliminary reaction is the adsorption of the enzyme upon the substrate, following which the enzyme attacks and alters the substrate. Here again, the illustration, while humorous, is at the same time graphic. Bayliss states:

" A somewhat trivial illustration of the phenomena of heterogeneous catalysis may be of service in understanding the process. Take the ' reaction ' between a strawberry and a number of snails in its neighbourhood. As soon as a snail, in its wanderings, becomes sensible of the presence of food, it proceeds towards it. This is the first, preliminary, stage of diffusion. The next stage, that of adsorption, may be represented by the attachment of the animal to the strawberry. This takes place rapidly, as soon as proximity is achieved. So long as nothing more happens, no chemical change results. The final stage is the devouring of the fruit and its consequent hydrolysis, etc. This final stage is obviously dependent, as far as its rate goes, upon the number of snails 'adsorbed.' It will also be noted that it will not be in linear proportion to the number of snails at work. The more there are, the more they interfere with one another, and, when the fruit is completely covered by them, the advent of more will not further accelerate the disappearance of the food, since the newcomers will not be able to get at it."

Two general formulae have been suggested as representing mathematically the characteristics of an adsorption reaction. Langmuir[55] has proposed the formula:

$$Q = \frac{abP}{1 + aP} \tag{74}$$

as representing an adsorption reaction in a gas-solid system where the gas is adsorbed upon the surface of the solid,

where Q = the amount adsorbed;
P = the equilibrium pressure of the gas in the system;
a and b = constants.

[54] Bayliss, W. M., The Nature of Enzyme Action, Fifth Edition, Longmans, Green and Company, New York (1925).

[55] Langmuir, I., The Adsorption of Gases on Plane Surfaces of Glass, Mica, and Platinum, *J. Am. Chem. Soc.*, 40 : 1361–1403 (1918).

Freundlich[56] has proposed the general adsorption formula:

$$\frac{x}{m} = aC^b \qquad (75)$$

where x = the weight of substance adsorbed, in grams;
m = the weight of adsorbent, in grams;
C = the concentration of the solution at equilibrium;
a and b = constants depending on the nature of the adsorbent and the substance which is adsorbed.

Freundlich's formula is a logarithmic expression and may be written:

$$\log \frac{x}{m} = \log a + b \log C \qquad (76)$$

Formula (75) is the mathematical expression of a parabola. The characteristic feature of the curve is that there is no single point where the reaction appears to be completed or where a new reaction begins. The curve is a sweeping parabola, and the system varies continuously and represents a reversible reaction.

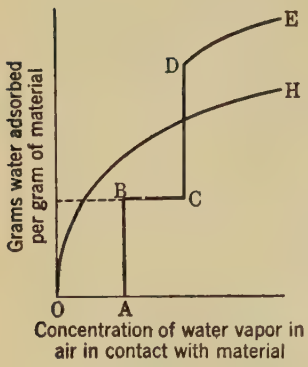


FIG. 60.—Contrasting the form of curves characteristic of surface phenomena with that characteristic of a chemical reaction. (After Bancroft.)

Surface phenomenon: Curve *O–H*—The adsorption of water vapor by dry charcoal.

Chemical reactions: Curve *A–B–C–D–E* — Behavior of Na_2SO_4 to water vapor.

O–A = anhydrous Na_2SO_4.
A–B = anhydrous Na_2SO_4 in contact with $Na_2SO_4 \cdot 10\ H_2O$.
Point B = only $Na_2SO_4 \cdot 10\ H_2O$.
C–D = $Na_2SO_4 \cdot 10\ H_2O$ in contact with a saturated solution of Na_2SO_4.
Point D = a saturated solution of Na_2SO_4.
D–E = varying concentrations (less than saturated) of Na_2SO_4.

Figure 60 gives a diagrammatic representation of an adsorption curve (*OH*) as contrasted with a chemical reaction (curve *ABCDE*). In the curve (*OH*) we have a reaction such as occurs when water vapor is adsorbed upon the surface of dry charcoal or some other inert surface. In curve (*ABCDE*) we have a curve characteristic of the behavior of anhydrous sodium sulfate toward water vapor.

In Fig. 61 are shown two curves drawn from the experimental data of Newton and Gortner,[57] representing the "binding" of water on the

[56] Freundlich, H., Kapillarchemie, Akademische Verlagsgesellschaft, Leipzig (1909).

[57] Newton, R., and Gortner, R. A., A Method for Estimating the Hydrophilic Colloid Content of Expressed Plant Tissue Fluids, *Bot. Gaz.*, 74 : 442–446 (1922).

surface of the hydrophilic colloid, gum acacia. Curve (*A*) represents the arithmetical values of the experiment, where the abscissa represents the per cent of gum acacia in the system, the ordinate the per cent of water in the system, which was bound. When the arithmetical values were converted into logarithms, curve (*B*) resulted. It will be noted that curve (*B*) forms a straight line which is one of the tests as to

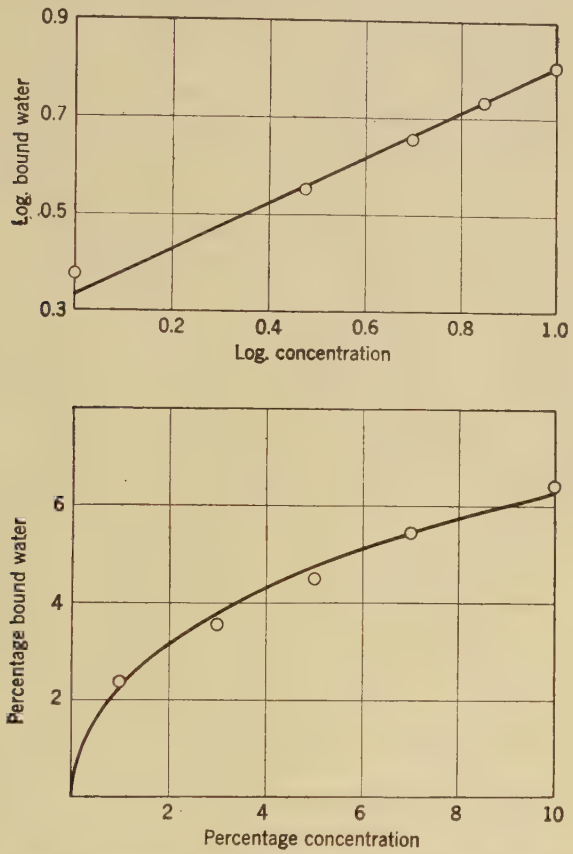


FIG. 61.—The relation between the concentration of gum acacia sols and the amount of water bound by the colloid. (Data of Newton and Gortner.)

whether or not a given reaction is an adsorption reaction or a chemical reaction. Many chemical reactions appear to follow a parabolic curve. When, however, the data of such a reaction are plotted in terms of logarithms, the resulting curve is not a straight line. If the resulting curve is a straight line, we can be fairly sure that we are dealing with an adsorption reaction.

The constants (a) and (b) of the Freundlich adsorption isotherm can be determined by plotting, in terms of logarithms, the results of a reaction. The value of (a) is the value on the ordinate axis, where the straight line cuts the axis of ordinates at unit concentration, as shown in Fig. 62. The constant (b) is an expression denoting the slope of the curve and is the tangent of the angle (θ) which the line makes with a line drawn parallel to the axis of abscissa. It is an expression for the rate of adsorption

$$\frac{d \log x/m}{d \log C}$$

when all variables except concentration have been held constant. Accordingly it is not necessary to plot the logarithmic curve in expressing graphically an adsorption reaction. One need only tabulate the values for these two constants and anyone can at leisure draw the characteristic curve. Hoffman and Gortner[58] have used this method in presenting a very extensive series of data.

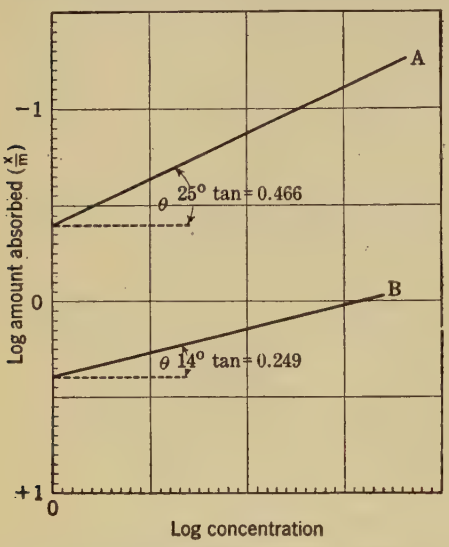


FIG. 62.—Showing the significance of the constants in the expression,

$$\frac{x}{m} = aC^b \quad \text{or} \quad \log \frac{x}{m} = a + b \log C.$$

Curve A,

$$\log \frac{x}{m} = 0.4 + 0.466 \log C.$$

Curve B,

$$\log \frac{x}{m} = -0.4 + 0.249 \log C.$$

In case one does not wish to use the graphic method which yields at the best only a close approximation for the value of these fundamental constants, but wishes instead to determine their absolute value, the method of least squares can be employed for their calculation. Denoting values on the axis of ordinates as (y) and values on the axis of abscissa as (x),

$$a = \frac{\Sigma(x) \cdot \Sigma(xy) - \Sigma(x^2) \cdot \Sigma(y)}{[\Sigma(x)]^2 - n\Sigma(x^2)} \tag{77}$$

$$b = \frac{\Sigma(x) \cdot \Sigma(y) - n\Sigma(xy)}{[\Sigma(x)]^2 - n\Sigma(x^2)} \tag{78}$$

[58] Hoffman, W. F., and Gortner, R. A., Physico-Chemical Studies on Proteins. I. The Prolamines—Their Chemical Composition in Relation to Acid and Alkali Binding, Colloid Symposium Monograph, Vol. II, pp. 209–368, Chemical Catalog Company, Inc., New York (1925).

where Σ = the sum of the numbers involved;
n = the number of individual items which were summed.

Much has been written in regard to the forces which are operating in the surface to cause these adsorption effects. One group of workers has taken the viewpoint that the reactions are purely physical and are occasioned by changes in surface tension, electrical forces, etc., while others have insisted that the forces are those of chemical union in which the forces of primary valence are operating. Probably both are correct, the predominance of one type or the other being determined by the system which is being studied.

It would be very difficult to combat the argument of those who insist that chemical reactions are the predominating factor, if it were not for the fact that the inert gases undergo characteristic adsorption reactions. Argon or radon, for example, can be rather readily adsorbed upon a charcoal surface. It is impossible to picture the interaction of primary valencies in such an adsorption. Accordingly in this case we are fairly safe in postulating a purely physical reaction.

On the other hand, we can very easily picture a mechanism whereby a chemical reaction will be proportional to the surface area of the solid material. Such a system is shown diagrammatically in Fig. 63, where a hypothetical cross section of a fragment of charcoal is pictured. It will be noted that the carbon atoms in the interior of the plane have all four of their valences satisfied by attachment to other carbon atoms, but the atoms on the edges and especially on the corners of the plane possess certain "free" valences which presumably are free to unite with or to attract other substances in their immediate vicinity. It is equally obvious that if we were to increase the surface of this hypothetical plane by again sectioning it through the center, we would increase the number of free valences, although the actual mass of carbon remained the same. As a result we

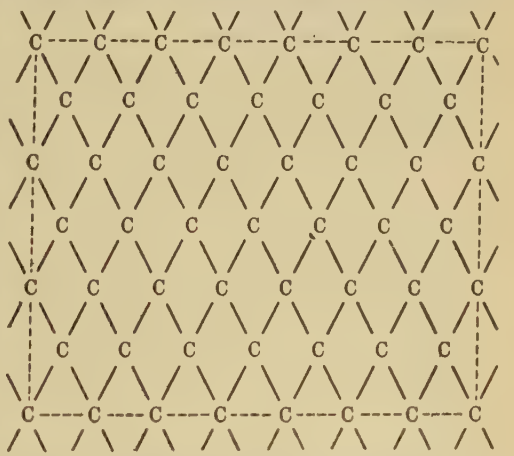


Fig. 63.—A diagrammatic representation of the unsatisfied valences at the sides and corners of a plane surface of space-oriented carbon atoms.

would increase the surface activity of a unit mass of carbon by increasing the surface area, and those who argue for a chemical reaction could legitimately say that the adsorption which took place was due to valence forces. However, a stoichiometrical equation could not be written for such a series of reactions, inasmuch as the reaction between a unit mass of carbon and a material which was adsorbed would depend not on the weight of the carbon but rather on the specific surface of the carbon. Probably many of the reactions which are characteristic of colloidal systems involve the forces of primary valence residing in surfaces.

It is to Taylor[59] that we owe this expression of active surface valences. In discussing catalysis he points out that Blench and Garner[60] have obtained values for the heat of adsorption of oxygen on charcoal, ranging from 50,000 cal. to 224,000 cal., whereas the heat of combustion of solid carbon to carbon dioxide is only 94,000 to 97,000 cal. He notes that if in the very finely divided carbon, certain of the carbon atoms were practically free at the corners and edges of the main mass of carbon, very few bonds of primary valence would have to be broken, and accordingly the union of oxygen with such atoms of carbon would be very similar to the union of oxygen with carbon in a gaseous state. Gaseous carbon and oxygen should give a very high heat of combustion which he calculates would be in the neighborhood of 350,000 cal. Taylor, accordingly, notes that the values obtained by Blench and Garner are intermediate between the values for combustion of solid and gaseous carbon atoms and furnish experimental evidence for the existence, at isolated spots on the surface, of atoms which approximate a gaseous state.

The theories which are involved in these considerations are of fundamental importance to anyone who is interested in adsorption reactions or in processes involving catalytic phenomena. Taylor notes that in the hydrogenation of oils, using a nickel catalyst, only a very small area (< 1 per cent) of the surface of the catalyst is actually active and suggests that the activity of this portion of the surface may be due to nickel atoms which have a number of free valences, so that even at room temperature their potential activity approaches more nearly gaseous nickel than massive nickel.

Adsorption may be either positive or negative. *Positive adsorption from solution is the concentration of the solute in the interfacial film.* This

[59] Taylor, Hugh S., Fourth Report of the Committee on Contact Catalysis, *J. Phys. Chem.*, 30 : 145–171 (1926).

[60] Blench, E. A., and Garner, W. E., The Heat of Adsorption of Oxygen by Charcoal, *J. Chem. Soc.*, London, 125 : 1288–1295 (1924).

concentration may reach high values and cause a very marked difference in the concentration of the original solution.

Negative adsorption is the concentration of the solvent in the surface film, leaving an excess of the solute in the body of the liquid. Negative adsorption is always small in amount. Apparently in most cases only a monomolecular film of solvent is adsorbed at the interface. We have already noted that Harkins and McLaughlin measured the thickness of the pure water film on the surface of salt solutions and found it to be a monomolecular layer.

The nature of the solvent is of very great importance in determining not only in many cases whether or not adsorption will take place, but also the extent of the adsorption reaction. As a general rule, *adsorption is the greatest from solvents which have a high surface tension and least from solvents which have a low surface tension.* This is what one would expect, considering the fact that surface energy interchanges are involved.

Willard Gibbs[61] predicted, long before experimental evidence was available, that substances which lower the surface tension or the interfacial tension would tend to concentrate in the surface film, because of the decrease in surface energy which would result. Gibbs' equation may be written as follows:

$$C_2 = -\frac{C}{RT}\cdot\frac{d\gamma}{dC} \tag{79}$$

where C_2 = the excess concentration in the interface;
C = the equilibrium concentration in the liquid phase;
dC = the increment of change in concentration of the solution for an increment of change $(d\gamma)$ in the interfacial tension.

(C_2) is positive when $\left(\frac{d\gamma}{dC}\right)$ is negative, or in other words, when the interfacial tension is decreased. Under such conditions we have positive adsorption. (C_2) is negative when $\left(\frac{d\gamma}{dC}\right)$ is positive or when there is an increase in the interfacial tension of the system. Under such conditions we would have negative adsorption.

Referring again to formula (75) it will be seen that the amount of material adsorbed is in direct proportion to the weight of the adsorbent, on the assumption that a portion of the same adsorbent in the same physical state is used. It would probably be more correct to say that *the amount of material adsorbed is directly proportional to the specific sur-*

[61] The Scientific Papers of Willard Gibbs, Vol. I, Thermodynamics, Longmans, Green and Company, New York (1906).

face of a given adsorbent. The measurement of specific surface is extremely difficult. In many instances it is practically impossible to determine the surface area of a given solid. Since, however, the amount of material which is adsorbed is proportional to extent of surface of a given material, we can utilize adsorption studies in determining the relative surface area of different samples of the same given material. Thus, for example, Holmes and Anderson[62] have devised a method for preparing silica gels having different specific surfaces. These gels were prepared by precipitating the silica gel in the presence of iron salts, drying the gel, and then leaching out the iron with acids, followed by a final washing. Holmes determines the relative surface areas of the various gels by using them as adsorbents for the vapors of organic liquids, the gel having the greatest specific surface adsorbing the greatest amount of organic vapor. Providing the substances are identical in all properties except extent of surface, the relative extent of surface can be determined through adsorption studies.

Garner, McKie, and Knight [63] have used adsorption methods to determine the internal surface of charcoal and find the surface area of one gram to be 66 square meters. Paneth and Radu[64] found values for wood charcoal ranging from 46.2 to 268.0 square meters. Harkins and Ewing,[65] in a very careful study of the density and volume relations of charcoal, reached the conclusion that the internal area of 1 gram of charcoal is probably somewhat less than 120 square meters. Incidentally they note in this paper that the intensity of adsorption of water on this charcoal surface is so great that the liquid is compressed to about 75 per cent of its original bulk, and they calculate that in order to bring about this compression a pressure in the neighborhood of 37,000 atmospheres would be necessary. This point will be referred to again.

Gustaver,[66] on the other hand, obtained much higher values of surface area. He considers the structure of charcoal as that of a mass of capillary spaces and that the adsorption of a vapor by charcoal consists of two stages, (*a*) the adsorption of the vapor at the interface, and (*b*) the condensed vapor then collecting in the form of liquid in the capillary

[62] Holmes, H. N., and Anderson, J. A., A New Type of Silica Gel, *Ind. Eng. Chem.*, 17 : 280–282 (1925).

[63] Garner, W. E., McKie, D., and Knight, B. C. J. G., The Area of the Internal Surface of Charcoal as Determined by the Adsorption of the Normal Aliphatic Alcohols from Aqueous Solution, *J. Phys. Chem.*, 31: 641–648 (1927).

[64] Paneth, F., and Radu, A., Über die Adsorption von Farbstoffen an Diamant, Kohle und Kunstseide, *Ber.*, 57 : 1221–1225 (1924).

[65] Harkins, W. D., and Ewing, D. T., A High Pressure Due to Adsorption, and the Density and Volume Relations of Charcoal, *J. Am. Chem. Soc.*, 43 : 1787–1802 (1921).

[66] Gustaver, B., Die Sorption von Dämpfen durch Kohle, *Kollch. Beihefte*, 15 : 185–338 (1922).

pores. Because of this second stage, the equilibrium is not reached instantaneously. He concludes that 1 gram of charcoal possesses 600 square meters of surface area in the form of capillaries greater than 6×10^{-8} centimeters in radius and that when the finer capillaries are taken into consideration, the total surface area of 1 gram of charcoal may easily exceed 3000 square meters.

Apparently the nature of the surface of a solid phase may determine in a large degree its behavior in acting as an adsorbent. This difference in behavior is probably determined in part by the magnitude of the electrokinetic potential residing in the surface. However, this suggestion is as yet only a hypothesis. We do know that in order for charcoal to become an efficient adsorbent it must be "activated." The activation of charcoal is carried out in several ways, one of the commoner ones being to heat the charcoal for a time in a current of steam and later heat in a closed container to 700 to 800° C. No adequate proof has as yet been secured to explain the processes that take place in such "activation," but undoubtedly changes in the surface do take place.

Effront[67] notes some very interesting experimental observations on the adsorption of various materials by proteins, agar agar, and vegetable tissues in general. Perhaps the most striking part of his data deals with the adsorption of pepsin by pure cellulose (filter paper prepared for quantitative analysis). One would suppose *a priori* that filter paper made by different manufacturers would have identical adsorptive properties. Table XXIII shows, however, that Effront found them to behave very differently toward pepsin.

TABLE XXIII

Adsorption of Pepsin by Filter Papers of Various Manufacturers

Manufacturer	Units of Pepsin Adsorbed	Adsorption, Per Cent	Manufacturer	Units of Pepsin Adsorbed	Adsorption, Per Cent
Laurent	0	0	Dreverhoffs, No. 417	165	50
Berzelius	33	10	Dreverhoffs, No. 402	297	90
Schleicher, No. 589	108	33	Dreverhoffs, No. 311	330	100
H. J. M. 100	128	39			

Such experiments indicate the errors which might be introduced into a study of the quantitative behavior of proteolytic enzymes when solutions which have been filtered through filter paper are used for enzyme

[67] Effront, Jean, Contribution à l'étude du pouvoir adsorbant des tissus végétaux, *Ann. et Bull. Sci. Med. Nat. Bruxelles*, 1926 (4) : 24–178 (1926).

study. Dreverhoff's paper No. 311, Effront notes, adsorbed all the pepsin from a given solution and allowed the accompanying contaminating proteins to pass through. On the other hand, the Laurent filter paper adsorbed selectively the proteins and allowed all of the pepsin to pass through.

Briggs,[68] in a paper already cited, has shown that celluloses of different origin and of different treatment have different electrokinetic potentials. Whether or not the factor operating in Effront's experiments was a difference in the electrokinetic potential or whether the behavior was due to other as yet unknown factors can be determined only by further study. Effront points out, however, that neither the ash content nor any chemical analysis of the paper which he could make would account for their different adsorptive behaviors. Using a Laurent filter paper, Effront was able to selectively adsorb practically all of the contaminating non-enzymatic impurities and states that by so doing he was able to prepare a pepsin which contained only 0.4 per cent nitrogen. He further notes that the enzyme, ptyalin, showed somewhat similar differential adsorption with the various papers.

The nature of the solvent is likewise of very great importance in adsorption reactions. This must be obvious when we take into consideration the Gibbs' equation (79) where changes in surface tension or interfacial tension account for the energy involved in adsorption processes. As we have already noted, adsorption is greatest from solvents which have a high surface tension and least from solvents which have a low surface tension, due to the fact that a greater change $\left(\frac{d\gamma}{dC}\right)$ can be brought about in a dispersions medium having a high surface energy than can be brought about in one having a low surface energy value. In many instances a substance will be positively adsorbed from water and be only slightly or not at all adsorbed from an organic solvent. Thus, for example, picric acid dissolved in water is very readily adsorbed on a charcoal surface, and can be removed completely from the aqueous phase by filtering off the charcoal. This charcoal-picric acid adsorption complex can be washed with large volumes of water without any appreciable quantity of picric acid appearing in the wash water. If, however, the charcoal-picric acid adsorption complex is washed with alcohol or with ether, the lower surface tension of the solvent causes the alcohol or the ether to replace the picric acid in the charcoal-water interfacial film, and the displaced picric acid moves out of the interface, giving a yellow solution in the alcohol or ether outside.

This reaction is of particular importance in the purification of organic

[68] Briggs, D. R., *J. Phys. Chem.*, 32 : 641–675 (1928).

compounds. It is often necessary to "bone-black" a solution of an organic compound in order to remove contaminating impurities. Decolorizing a solution with bone-black or an activated vegetable carbon is a very common example of selective adsorption. After satisfactory decolorization has been secured, it often happens that the student washes, with an organic solvent, the carbon which has been filtered off, in order to be sure that he is not losing any appreciable amount of the valuable constituent which he wishes to conserve. In many instances these wash liquors are highly colored and contain the impurities which he originally wished to remove. Thus, the washing of the carbon adsorption complex with an organic solvent may entirely defeat the original purification program.

On the other hand, it has in a number of instances been possible to concentrate on an interface by the process of adsorption all of the valuable constituent which is present in a solution, and then by later treating the adsorption complex with the proper solvent to completely remove the valuable constituent and obtain it in a highly concentrated and a relatively pure condition. Thus, for example, alkaloids can be quantitatively removed from an acid solution by "Lloyd's reagent"[69, 70] (hydrous aluminum silicate). The affinity of Lloyd's reagent for alkaloids is so great and such complete adsorption takes place that no bitter taste can be detected either in the mother liquor or in the Lloyd's reagent-alkaloid adsorption complex, when such substances as quinine or strychnine are adsorbed. The adsorption complex can be filtered off and washed thoroughly with water so as to get rid of all contaminating sugars, salts, etc., which may have been present in the original extract. If now this adsorption complex is shaken with a slightly alkaline aqueous solution, the alkaloid which was adsorbed upon the surface is quantitatively liberated and can be extracted from the solution by shaking out with chloroform, ether, etc. Similar examples of selective adsorption are numerous.

The adsorptive process is so general that care should be taken at every step in the preparation or the quantitative estimation of biochemical compounds in order to prevent adsorption reactions from interfering with the results which are desired. Thus, for example, Bock[71] notes that it is an almost universal procedure to decolorize solutions with finely divided carbon. He notes that when urine was decolorized with

[69] Lloyd, J. U., Process of Extracting, Purifying, or Excluding Alkaloids and Alkaloidal Salts, U. S. Patent 1,048,712. Dated Dec. 31, 1912.

[70] Lloyd, J. U., Discovery of the Alkaloidal Affinities of Hydrous Aluminum Silicate, *J. Am. Pharm. Assoc.*, 5 : 381–390; 490–495 (1916).

[71] Bock, J. C., A Study of a Decolorizing Carbon, *J. Am. Chem. Soc.*, 42 : 1564–1569 (1920).

the vegetable carbon, " Norit," the various constituents present in the urine were adsorbed and removed with the coloring matter in appreciable amounts. The percentage of the various constituents which were adsorbed and removed were as follows: total nitrogen 12.9–18.8 per cent, ammonia nitrogen 0.67–4.59 per cent, urea nitrogen 8.20–11.21 per cent, creatinine 53.07–91.85 per cent, uric acid quantitatively adsorbed, phosphates 12.93–38.76 per cent, chlorides 0–5.71 per cent, glucose 0.84–5.45 per cent. He further notes that in a pure dilute glucose solution, from 16 to 26 per cent of the glucose could be removed by adsorption with 5 per cent " Norit," and points out that samples of urine cannot be decolorized with carbon if one is to obtain accurate quantitative values for the constituents which were originally present.

A further example similar to the observation of Bock, was made by Gortner and Holm,[72] where it is pointed out that protein hydrolysates that have been " bone-blacked " give entirely erroneous values for tyrosine and tryptophane content. Accordingly *in quantitative studies it is never justifiable to decolorize a solution with carbon or with any other adsorbent unless preliminary experiments have definitely proven that such decolorization does not remove that constituent which is being quantitatively estimated.* This is especially important in experiments where the material is estimated by colorimetric methods. In such instances it is, of course, essential that the original solution be colorless and clear, but many of the methods which are used to produce clear, colorless solutions of biological fluids introduce errors due to forces of adsorption.

The presence or absence of polar groups in the molecules of the solvent or of the solute may affect an adsorption reaction, probably due to their influence on the ease with which the surface of a solid adsorbent is wetted. Thus, for example, Traube's rule is in general true, as already indicated in the discussion of Fig. 7. Freundlich[73] extended the rule to adsorption from aqueous solution and states that *the adsorption of organic substances from aqueous solutions increases strongly and regularly as we ascend the homologous series.* Accordingly, in general, butyric acid is adsorbed more strongly than propionic, and propionic more strongly than acetic. However, *these generalizations are not invariably true.* Holmes and McKelvey[74] point out that Freundlich's adsorption studies dealt with carbon, a non-polar solid, in water, a polar liquid. They reversed the condition, using silica, a polar solid, and the various

[72] Gortner, R. A., and Holm, G. E., The Colorimetric Estimation of Tyrosine by the Method of Folin and Denis, *J. Am. Chem. Soc.*, 42 : 1678–1692 (1920).

[73] Freundlich, Herbert, Colloid and Capillary Chemistry, p. 195.

[74] Holmes, H. N., and McKelvey, J. B., The Reversal of Traube's Rule of Adsorption, *J. Phys. Chem.*, 32 : 1522–1523 (1928).

fatty acids dissolved in toluene, a non-polar liquid. They suggest that in Freundlich's studies the polar end (—COOH) of the fatty acid molecule was oriented toward the water phase and the hydrocarbon chain toward the carbon surface, whereas in their own studies, the highly polar carboxyl end would be oriented toward the silica surface and the non-polar alkyl group toward the hydrocarbon solvent. They found a reversal of Traube's rule and that acetic acid was very strongly adsorbed from toluene on silica gel, propionic acid less strongly adsorbed, butyric acid still less adsorbed, and caprylic acid relatively slightly adsorbed.

These observations form a rather striking example of the role that molecular orientation plays at interfaces.

The adsorption process, as represented by equation (75) *is an equilibrium, i.e.,* if an adsorbing surface is brought into contact with a solution which has an equilibrium concentration of 0.01 N, and the adsorbing substance is then removed from that solution and placed in a weaker solution which has an equilibrium concentration 0.001 N, the amount of material adsorbed will be identical with the amount that would have been adsorbed at an equilibrium concentration 0.001 N. Or conversely, if an adsorbing material is removed from the solution where the equilibrium concentration is 0.001 N and placed in a stronger solution so that the equilibrium concentration is 0.01 N, the adsorbing material will take up exactly the same amount as would have been taken up had it originally been placed in the stronger solution. Accordingly one cannot filter off an adsorbent which has reached equilibrium in a chemical solution and wash it with a solution that has a concentration different from the equilibrium solution without changing the amount of material adsorbed on the adsorbing surface. It is obvious, from the fact that an adsorption curve is a parabola, that proportionately greater quantities of material are adsorbed from the weaker solutions

Adsorption reactions are, in general, characterized by a positive heat of adsorption. In some instances the amount of heat liberated is large and indicates the great affinity existing between the surface of the adsorbent and the material being adsorbed. Harkins and Ewing[75] found that 1 gram of bone-charcoal gave a maximum heat of adsorption for water of 18.5 cal., and 1 gram of fullers' earth gave 32.0 cal. Keyes and Marshall[76] in a study of the heat of adsorption of various gases on charcoal

[75] Harkins, W. D., and Ewing, D. T., A High Pressure Due to Adsorption, and the Density and Volume Relations of Charcoal, *J. Am. Chem. Soc.*, 43 : 1787–1802 (1921).

[76] Keyes, F. G., and Marshall, M. J., The Heats of Adsorption of Several Gases and Vapors on Charcoal, *J. Am. Chem. Soc.*, 49 : 156–173 (1927).

find values as follows: ether 7250, ammonia 6456, carbon dioxide 5450, methane 4600 cal. per mole. They also have selected certain other measurements from the literature and record the following values for heats of adsorption: argon 3450, nitrogen 3654, hydrogen 1870, helium 387 cal. per mole. The values for helium are for a temperature of −134° C. instead of 0° C. to which all the other values refer.

This paper by Keyes and Marshall is of added importance, inasmuch

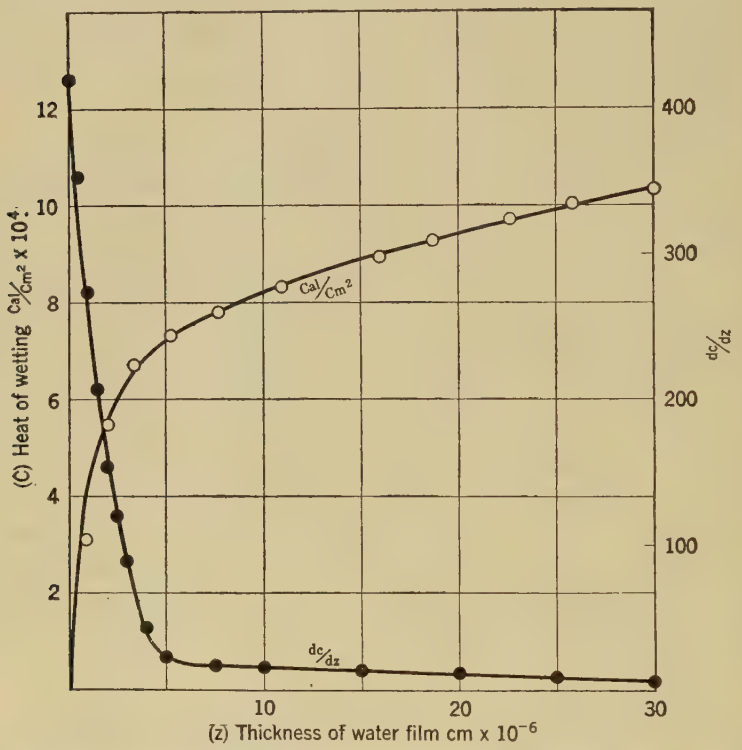


Fig. 64.—Thermal changes as related to the thickness of the water film on silica gel. (Data of Bellatti and Finazzi; Parks; and Nutting.)

as they point out that in the adsorption of gases one is not necessarily limited to a monomolecular layer. Their reasoning is that the initial reaction forms a monomolecular layer of adsorbed gases upon the surface of the adsorbent, but that, " the first and succeeding layers, because of their special state, constitute new adsorptive surfaces which may adsorb molecules of the same species as the first layer or molecules of a different species. Each succeeding layer (the adsorbing molecules all of the same species) partakes of the special state of the first layer in less-

ened degree until finally a layer is reached wherein the molecular state differs little from what may be imagined as a molecular 'contact' arrangement." This reasoning appears to be very logical, and we shall have occasion to refer to a similar hypothesis when we consider the solvation of lyophilic colloids.

Nutting[77] has used the data of Bellatti and Finazzi,[78, 79] together with certain calculations by Parks,[80] as shown in Table XXIV, to calculate the surface forces on a silica-water interface. He has likewise calculated the differential curve $\left(\frac{dc}{dz}\right)$, where (c) is the energy in calories evolved by the adsorption of water on a square centimeter $\times 10^4$ of the silica surface and (z) equals the thickness of the adsorbed water film in centimeters $\times 10^{-6}$. Figure 64 illustrates the curves which were obtained.

TABLE XXIV

Heat of Wetting of Silica of Varying Moisture Content

Calculations of Bellatti and Finazzi		Calculations of Parks		
A Adsorbed H_2O/gm. SiO_2	*B* Cal./gm. of SiO_2	*C* Cal./gm. 26.0 − B	*D* Depth of $H_2O \times 10^{-6}$ cm.	*E* Cal. per $cm^2 \times 10^{-4}$
0.00	26.0	0.0	0.0	0.0
0.0238	18.29	7.71	0.95	3.08
.0535	12.23	13.67	2.14	5.47
.0859	9.17	16.83	3.44	6.73
.1292	7.61	18.39	5.17	7.36
.1883	6.50	19.50	7.53	7.80
0.2736	5.25	20.75	10.94	8.30
.3995	3.70	22.30	15.98	8.92
.4635	2.94	23.06	18.54	9.22
.5648	1.66	24.34	22.59	9.74
.6478	0.90	25.10	25.91	10.04
.7694	0.19	25.81	30.78	10.32

[77] Nutting, P. G., The Adsorptive Force of Silica for Water, *J. Phys. Chem.*, 31 : 531–534 (1927).

[78] Bellatti, M., and Finazzi, L., Sul calore che si produce bagnando le polveri, *Atti. r. Ist. Veneto*, 61, II, 503–524.

[79] Bellati, M., Sul calore svolto nel bagnare le polveri, *Atti. r. Ist. Veneto*, 59, II : 931–947 (1900).

[80] Parks, I. J., On the Heat Evolved or Absorbed When a Liquid is Brought in Contact with a Finely Divided Solid, *Phil. Mag.*, (VI) 4 : 240–253 (1902).

He calls attention to the fact that the differential curve turns sharply at a thickness of water film between 4 and 5 $\times 10^{-6}$ cm., representing a layer of water 100–120 molecular diameters deep, and that the energy is not by any means confined to the first molecular layer, nor is there any apparent break after the first molecular layer has been formed. He calculates the maximum pressure at the surface to be of the order of 17,410 atmospheres and that if conditions at the surface were responsible for the adsorptive forces, the pressure should fall to zero at approximately 73 molecular diameters, and he adds that if the solid pulls on the adsorbed liquid with a pressure equal to 17,000 atmospheres, the liquid must also pull on the solid to the same degree and that accordingly *the pull of water on a quartz surface approximates the tensile strength of quartz!* This leads to a hydration and dispersion of quartz by water which is adsorbed onto the surface.

Patrick and Grimm[81] have measured the heat of adsorption of liquids on silica gel and give the following values: water 19.22, ethyl alcohol 22.63, benzene 11.13, carbon tetrachloride 8.42, and aniline 17.54 cal. per gram, and from these data they calculate a surface area of 6.9 $\times 10^6$ sq. cm. per gram of silica gel.

If heat is evolved by the adsorption of water upon a surface, at least an equivalent amount of heat must be added before the adsorbed water is released from the surface. Neuhausen and Patrick[82] point out that a silica gel can be heated to 300° C. in a vacuum produced by a Gaede pump for a period of six hours without reducing the water content below 4.8 per cent. This indicates that the adsorptive force of silica gel for water is so great that even at 300° C. in a vacuum, water would be positively adsorbed over the interface.

In general, *adsorption reactions are characterized by a negative temperature coefficient.* This is to be expected, providing there is a positive heat of adsorption. The fact that adsorption becomes less as the temperature increases is probably due, at least in part, to the increased kinetic energy of molecules with an increase in temperature. An adsorption reaction can accordingly, in some instances at least, be distinguished from a chemical reaction, in that a decrease in rate of adsorption and a decrease in the amount of material adsorbed occurs at a higher temperature, whereas a classical chemical reaction is characterized by an increase in rate with an increase in temperature, although in many instances the

[81] Patrick, W. A., and Grimm, F. V., Heat of Wetting of Silica Gel, *J. Am. Chem. Soc.*, 43 : 2144–2150 (1921).

[82] Neuhausen, B. S., and Patrick, W. A., Organogels of Silicic Acid, *J. Am. Chem. Soc.*, 43 : 1844–1846 (1921).

same equilibrium is reached. Accordingly the constants (*a*) and (*b*) of equation (75) may be different at different temperatures.

PSEUDO-ADSORPTION.—As already noted, true adsorption is an equilibrium, and diluting the system would shift the equilibrium concentration to the same point that would have been reached, had the reaction been carried out originally at the lower concentration. It occasionally happens, however, that what appears to be an adsorption reaction is not a true equilibrium and is not reversible. Thus, for example, if porous porcelain is placed in a solution of auric chloride, gold is adsorbed from the solution which cannot be subsequently dissolved out of the adsorbing surfaces. The reaction in this case is probably similar to the reaction which Latshaw and Reyerson[83] utilize to prepare metallized silica gels. They found that when silica gel was placed in solutions of salts of certain metals, including the noble metals, the salts were decomposed and the surface of the silica gel became covered with a thin layer of metal. They interpret this as a reduction of the salt by hydrogen (or other reducing substances) adsorbed on the surface of the silica gel. Similar reduction took place on the surface of charcoal. Apparently the initial reaction is the adsorption of the metallic salt at the interface, and this adsorption is followed by a chemical reaction whereby the metallic ions are reduced to a metal film. Accordingly the concentration of the metallic salt in the interface is altered, and additional adsorption takes place from the solution until finally by repetition of these processes the solution is almost entirely depleted of metallic ions.

A somewhat similar reaction occurs when an aqueous solution of egg albumin is beaten to a foam. The egg albumin is positively adsorbed at the gas-liquid interface and *the surface energy coagulates the adsorbed egg albumin in the same way that egg albumin can be coagulated by heat.* Such coagulated egg albumin will no longer dissolve in water, and accordingly we have an insoluble film of egg albumin which persists and prevents a reversal of the adsorption equilibrium. By stirring or bubbling air through a weak solution of egg albumin, the egg albumin can be removed practically quantitatively from the weaker solution, and if the foam is allowed to stand until it breaks and is then filtered, it is possible to collect nearly all of the original egg albumin in the form of insoluble films.

Pseudo-adsorption can, therefore, be defined as adsorption followed by a chemical change in the material which has been adsorbed, the chemical change being of such a nature as to inhibit the reversal of the adsorption process.

Pseudo-adsorption probably plays a role in the "poisoning" of

[83] Latshaw, M., and Reyerson, L. H., The Reducing Action of Hydrogen Adsorbed in Silica Gel, *J. Am. Chem. Soc.*, 47 : 610–612 (1925).

hydrogen electrodes. It occasionally happens that a hydrogen electrode behaves erratically and gives false values or gives potentiometric readings which "drift." In such instances the probable explanation is that some substance has been adsorbed on the electrode which has a greater affinity for the platinum surface than has hydrogen. Mercury, copper, arsenic, and various other substances will "poison" a platinum electrode. These metals are probably reduced by the hydrogen present on the electrode, so that we are no longer dealing with a platinum surface but rather with a mercury, copper, or arsenic surface which gives an erroneous or an unstable potential. Arsenic is notoriously poisonous to the platinum catalyst used in the contact process for the manufacture of sulfuric acid. Here again the explanation probably lies in the adsorption upon the platinum surface of arsenic or arsenic compounds, causing the surface to become catalytically inert.

Catalysis by Adsorption.—We have already noted Taylor's view in regard to the nature of an active surface of nickel used in hydrogenation, *i.e.*, that we have certain free valences at isolated points in the catalytic surface, where atoms of nickel behave more or less as gaseous nickel.

Almquist[84] takes a somewhat similar view of the nature of the surface of iron catalysts used in the synthesis of ammonia from nitrogen and hydrogen.

Boswell and Dilworth,[85] in a study of the role which aluminium oxide plays in the catalysis of certain organic reactions, such as the formation of ethylene and water from ethyl alcohol, point out that all of the reactions which are catalyzed by aluminium oxide involve either hydrogen or hydroxyl ions, or the addition of water to, or the removal of water from the reacting compounds, and reach the conclusion that aluminium oxide functions by means of a surface film of water which is the real seat of the catalysis.

In an attempt to determine how firmly this water film was held upon the surface of the aluminium oxide, they employed rather high temperatures, and conclude that "there is still a water film on the surface, even after heating at atmospheric pressure at 500° C. for twenty hours, followed by two days heating with a Meker burner." At the end of this period there was still 0.36 per cent of water present in the aluminium oxide. Because of the great stability of the water film, they conclude that the water is not present as water molecules but rather in the form of adsorbed hydrogen and hydroxyl ions. Their hypothesis of a water

[84] Almquist, J. A., The Nature of the Catalyst Surface and the Effect of Promoters, *J. Am. Chem. Soc.*, 48 : 2820–2826 (1926).

[85] Boswell, M. C., and Dilworth, H. M., On the Mechanism of Catalysis by Aluminium Oxide, *J. Phys. Chem.*, 29 : 1489–1506 (1925).

surface is shown diagrammatically in Fig. 65, and they suggest that it is this surface of positively charged hydrogen ions and negatively charged hydroxyl ions that is actually the seat of the catalytic activity. In regard to catalysis they state, " *The catalyst from this point of view does not accelerate a reaction already in progress . but actually initiates the change from one to the other.*"

As we shall see later, Taylor has made a similar statement that a catalyst may initiate a reaction because of the energy residing in the catalytic surface.

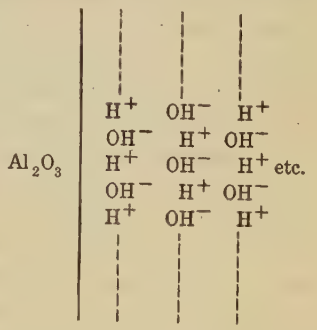


FIG. 65.—A possible space relationship of water adsorbed on the surface of aluminium oxide. The last layer of water is not driven off by heating at 500°C. for twenty hours, followed by heating for two days with a Meker burner. (After Boswell and Dilworth.)

SOME APPLICATIONS OF ADSORPTION.—The adsorption of gases on surfaces became of extreme importance when gas warfare was introduced. The problem of offense was to use poison gases which were not readily adsorbed upon the charcoal or other adsorbents used in the canisters of the gas masks, whereas the problem of defense was to quickly prepare suitable adsorbents for the gases which were introduced. In many instances, special adsorbents were found, and throughout the entire period of the World War, there was a rapid advance in the development of new adsorbents and improving the adsorbing power of the common materials, such as charcoal, silica gel, etc. Since the War the knowledge gained in this way has been applied to industrial problems in removing vapors of solvents, etc., from the atmosphere of factories, for the purpose of either reducing the health hazards or for recovery of valuable solvents.

In the manufacture of sulfuric acid by the contact process, adsorption plays a very important role. The contact catalyst is, as a rule, finely divided platinum sponge supported on asbestos or some other inert supporting surface. The oxygen and sulfur dioxide are adsorbed upon the platinum surface so strongly that they are brought within the radius of chemical attraction and unite to form sulfur trioxide which is less strongly adsorbed than the initial reacting materials. The platinum surface accordingly is supersaturated with respect to the sulfur trioxide. Sulfur trioxide leaves the surface, allowing room for additional adsorption of oxygen and sulfur dioxide. Such a cycle will continue indefinitely as long as the surface remains in its original condition. The " poisoning " of such a surface is very readily brought about by the

presence of certain heavy metals and notably by arsenic. The poisoning of the surface is undoubtedly due to the irreversible adsorption of some foreign material on the catalyst where it is held with a greater tenacity than are the products of the reaction which it is proposed to catalyze. That portion of the surface which is covered by the adsorbed poison film, therefore, becomes catalytically inert, and whenever any appreciable part of the catalyst surface becomes inert, the catalyst must be removed and reworked in order to remove the poisoning agent and prepare a surface which will be catalytically active.

Adsorption has been used with considerable success in the de-inking of magazines and old newspapers, in making the paper available for re-use as newsprint. The Forest Products Laboratory of the United States Forest Service has made use of bentonite for the selective adsorption of printer's ink from the re-pulped paper. Bentonite is apparently a decomposition product of volcanic ash characterized by an almost entire absence of quartz, and is a clay-like material containing relatively large amounts of alkaline oxides and alkaline earths, the particle size being very uniform and largely in the range of from 0.1μ to 0.5μ with relatively few particles larger than 1μ. Bentonite exhibits colloidal properties to a remarkable degree. The material, as mined, is a soft, flaky mineral which, if placed in water, swells to a remarkable extent, passing first into a gel and finally completely dispersing into a colloidal sol, the particles remaining in suspension for an indefinite period of time. The great surface area is probably responsible for its adsorptive efficiency. If pulped newspapers or magazines are mixed with a small amount of bentonite, the printer's ink is selectively adsorbed from the fiber by the bentonite, and when the mixture is run over wire screens and washed with water, the bentonite and the adsorbed printer's ink are readily washed from the fiber, yielding a reclaimed pulp which is practically ink-free and which can be made into a satisfactory sheet of paper with very little loss of the original fiber content. In this operation we have a rather striking example of selective adsorption.

The phenomenon of selective adsorption or rather of the wettability of surfaces by liquids is strikingly illustrated in the ore flotation process. Sulfide ores, such as copper sulfide, lead sulfide, etc., have a non-polar surface and are not easily wetted by polar liquids, such as water. On the other hand, they are rather easily wetted by non-polar liquids, such as oils. Silica and silicious minerals, on the other hand, having a polar surface, are readily wetted by water but are not readily wetted by oils. If a rock, containing small amounts of valuable sulfides intermixed with large amount of silicates, is finely ground and the ground mixture agitated with water to which a certain amount of oil has been added, the

oil is selectively adsorbed by the sulfide particles, so that they become coated with a film of oil, or a number of the sulfide particles adhere to a single oil droplet. If air is then blown through this mixture, the finely ground sulfides adhering to the oil particles rise to the surface in a foam which can be skimmed off, the silica and silicates of the gangue settling to the bottom of the agitator tank. In this way a relatively small amount of a valuable mineral may be separated from a relatively large amount of inert material. A concentrated high-grade ore is thus obtained. The ore flotation process has made it possible to utilize ores of much lower grade than was previously feasible. As a matter of fact it has made possible the working of large masses of residues which had been earlier discarded. More than 60,000,000 tons of ore are concentrated in the United States each year by the flotation process.

THE ADSORPTION OF WATER ON BIOLOGICAL PRODUCTS.—The lyophilic colloids, characteristic of biological systems, possess a great affinity for water. Some of the phenomena which are involved will be discussed later when we consider the problem of gels. At this point it is desired to call attention only to the problems which are involved in the drying of biological materials.

The determination of the moisture content of a biological material is a purely empirical procedure determined by the three variables, temperature, pressure, and time. It is impossible to make a definite statement that such and such a biological material has such and such a moisture content, without defining the conditions in regard to temperature, pressure, and time of drying under which the moisture determination was carried out. *The removal of water from a bio-colloid is merely shifting one equilibrium between a colloid surface and water to a new equilibrium,* and the extent to which the equilibrium is shifted is determined by these three variables.

Nelson and Hulett[86] have in particular called attention to this problem. They point out that if a cubic centimeter of organic material be divided into cubes 10^{-6} cm. on an edge, the area on the faces of these cubes would be equivalent to 600 square meters. Assuming a monomolecular layer of water 3.1×10^{-8} cm. thick on the surface of these cubes, there would be 0.186 cc. of water in the one cubic centimeter of material or approximately 15 per cent of the weight of the substance. This water would be in a condensed condition and would show practically no vapor pressure. If the layer of water were one-tenth as thick as the diameter of the assumed cubes, it would make up 50 per cent of the weight of the material and still have a vapor pressure considerably

[86] Nelson, O. A., and Hulett, G. A., The Moisture Content of Cereals, *J. Ind. Eng. Chem.*, 12 : 40–45 (1920).

less than that of normal water. Accordingly a part of the water would be removed by heating at atmospheric pressure at 100° C., more would be removed by heating in a vacuum at 100° C., and still more would be removed at each increment increase of temperature above 100° C. up to a point where decomposition of the organic material would set in.

The apparatus which they devised allowed them to differentiate between adsorbed water and water formed by the decomposition of carbohydrate or protein materials. They point out that above 365° C. which is the critical temperature of water, water cannot exist as a liquid no matter how much pressure is applied. Accordingly all water should be removed from the surface before that temperature is reached.[87] Table XXV shows some of the data which Nelson and Hulett obtained and illustrates the empirical nature of the moisture determination.

TABLE XXV

Showing the Difference between the Moisture Content of Various Biological Products as Obtained by the "Official" Method and the Probable True Moisture Content (Data of Nelson and Hulett)

	Apparent Water Content at 100° *in Vacuo*, Per Cent	Probable True Water Content, Per Cent	Difference Due to Water Films Having No Appreciable Vapor Pressure at 100° *in Vacuo*, Per Cent
Wheat Flour	10.80	11.80	1.00
Cornmeal	11.34	12.25	0.91
Cornstarch	11.80	12.40	0.60
Cellulose (Swedish filter paper)	2.63	2.80	0.17
Cellulose (absorbent cotton)	5.49	5.90	0.41
Protein (edestin)	10.40	12.30	1.90

Enzymatic Activity.—Apparently the first reaction between an enzyme and its substrate is an adsorption of the enzyme by the substrate. This is followed by the chemical action of the enzyme. The

[87] It should be pointed out that this argument depends upon the hypothesis that the water in an adsorbed film is present in the molecular state (H_2O) and not in the form of adsorbed and oriented hydrogen and hydroxyl ions as postulated by Boswell and Dilworth (see p. 182). The observations of Boswell and Dilworth make it very evident that adsorbed water can exist above 365° C., so that this particular argument of Nelson and Hulett needs some revision.

initial adsorption reaction can be well illustrated by the reaction which takes place between fibrin and pepsin. If a preparation of pepsin is added to a suspension of fibrin in 0.2 per cent hydrochloric acid, and the mixture is vigorously shaken and immediately filtered, it will be found that the pepsin has even in that short time interval been quantitatively adsorbed by the fibrin. This can be proven by the addition of fresh fibrin to the acid filtrate, under which conditions no appreciable digestion of the fibrin will be observed. If fresh 0.2 per cent hydrochloric acid is added to the fibrin granules which were filtered off, hydrolysis proceeds, and the fibrin will become completely digested. The initial combination of the enzyme with the substrate usually takes place almost instantaneously, the rapidity of reaction and other characteristics indicating rather conclusively that we are dealing with a purely adsorption phenomenon.

CHAPTER VII

ELECTROLYTES AND COLLOID SYSTEMS

We have already noted that a colloid micelle is stabilized by an electric double layer and that when the electric charge is neutralized and the micelle becomes isoelectric, flocculation occurs. We would accordingly expect solutions of electrolytes to have a very marked effect upon the properties of colloidal systems. Those colloids which are *negatively charged* are rendered isoelectric and are flocculated by the *cations* of an added electrolyte. whereas *positively charged* micelles are sensitive to the added *anions.*

Referring again to Fig. 6, it will be seen that the addition of Br^- causes the flocculation of positively charged silver bromide micelles until the isoelectric point is reached, following which a further addition of Br^- imparts a negative charge to the isoelectric silver bromide with the formation of negative silver bromide micelles. Accordingly as an electrolyte is added to a colloidal sol, the electrokinetic charge is progressively decreased to zero and then increased with further addition, forming a new sol possessing a charge opposite to that possessed by the original sol.

In general, the flocculating power of an ion follows the Hardy-Schulz rule that "*the precipitating power of an electrolyte depends upon the valency of the ion whose charge is opposite to that on the colloidal particle.*"

While the Hardy-Schulz rule is not a hard and fast rule, it is in general true, and differences between ions of the same valency can probably be explained by the differences in degree of hydration of the ions, differences in ionic mobility, and differences in the degree to which they are adsorbed on the surface of the colloid micelle.

Negatively charged sols are coagulated by the cations, Na^+, Ca^{++}, Al^{+++}, and are but relatively slightly affected by the anions, Cl^-, $SO_4^=$, $PO_4^{\equiv}$, the reverse being true for the positively charged sols. *The influence of valency is not an arithmetical* 1 : 2 : 3 *ratio but more nearly a geometrical progression* $1 : x : x^2$, where, in some instances at least, (x) has a value lying somewhere between 16 and 32. If (x) should have a value of 32, it would mean that Ca^{++} would be 32 times as efficient a flocculating agent as Na^+ and that Al^{+++} would be 1024 times as effective as Na^+.

TABLE XXVI

Showing the Effect of Electrolytes in Flocculating Ferric Hydroxide Sols (Data of Hardy) and Arsenous Sulfide Sols (Data of Freundlich)

$Fe(OH)_3$ Sol (Positive) Coagulated by Negative Ions			
NaCl	required	0.5 gm.	equivalents per liter concentration
K_2SO_4	required	0.0006 gm.	equivalents per liter concentration
H_2SO_4	required	0.0020 gm.	equivalents per liter concentration
HCl	required	0.5 gm.	equivalents per liter concentration

As_2S_3 Sol (Negative) Coagulated by Positive Ions			
NaCl	required	51.0	millimols per liter
LiCl	required	58.5	millimols per liter
KCl	required	49.5	millimols per liter
$MgCl_2$	required	0.717	millimols per liter
$MgSO_4$	required	0.810	millimols per lites
$CaCl_2$	required	0.649	millimols per liter
$BaCl_2$	required	0.691	millimols per liter
$AlCl_3$	required	0.093	millimols per liter
$Al(NO_3)_3$	required	0.095	millimols per liter

Table XXVI shows the relative amounts of various electrolytes required to flocculate a positively charged ferric hydroxide sol and a negatively charged arsenous sulfide sol. An inspection of the data in this table will make it clear why alum or iron sulfate are used in water purification processes. In our large cities the water is frequently taken from a muddy river, pumped through a station where chlorine is added to destroy microorganisms and where iron or aluminum sulfate is added to flocculate the suspended particles. The water then goes on to the filter beds and passes through the filters into the water mains. Relatively small amounts of Al^{+++} or Fe^{+++} are required in order to clarify such waters. The clarification could be conducted by the addition of sodium chloride, but the amount of Na^+ that would have to be added to produce the same flocculation would be so great that the water passing into the city main would probably taste salty, due to the excess of sodium chloride remaining in the water.

Deltas are formed where rivers carrying clay and silt meet the salt water of the ocean, the clay and silt being deposited because of the neutralization of their electric charge by the electrolytes in the ocean water.

There appears to be but little doubt among workers that the flocculating effect of electrolytes acting upon lyophobic colloids is due almost

entirely to the effect on the stabilizing electrokinetic potential. Kruyt,[1] and Kruyt and van Arkel[2] have shown that the electrokinetic potential does not have to drop to zero before the sol becomes unstable, but that instead there is a *critical zone* in the vicinity of the isoelectric point where the magnitude of the electric charge is not sufficiently great to insure indefinite stability. In other words, the electrokinetic potential must

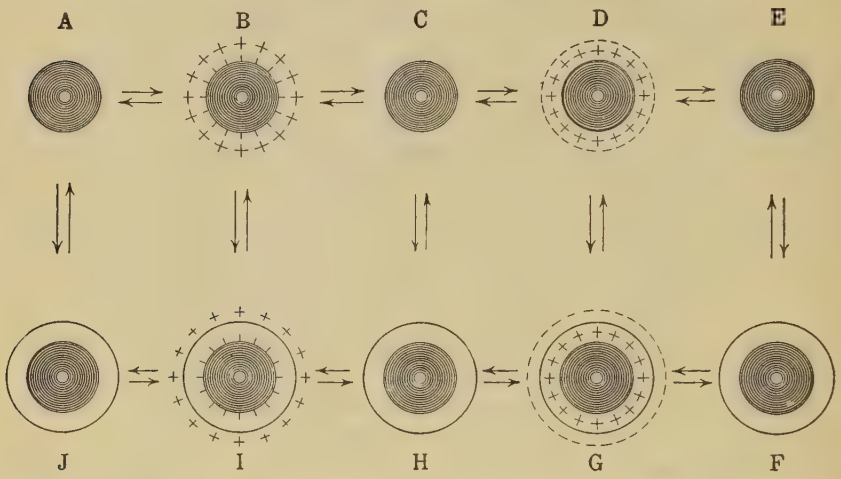


FIG. 66.—A diagrammatic representation of the relationships which exist between the solvation of a particle and the electrical charge of a particle, showing that both are important as factors influencing stability. *A–B–C–D–E* are lyophobic particles of which only *B* and *D* will exist in stable sols. *F–G–H–I–J* are lyophilic particles. All will be relatively stable as contrasted to the lyophobic series, but *G* and *I* have two factors for stability, solvation, and electric charge. Suitable electrolytes in low concentration will neutralize or even reverse the charges. "Desolvating" agents (*e.g.*, alcohol for water systems) will convert the solvated particles to lyophobic systems. (After DeJong.)

reach a certain minimal value before it confers stability on a colloid micelle.

De Jong,[3, 4] working in Kruyt's laboratory, has shown that lyophilic

[1] Kruyt, H. R., Sur le potentiel critique, *Rec. trav. chim. Pays-Bas*, 39 : 618–622 (1920).

[2] Kruyt, H. R., and van Arkel, A. E., Sur la relation entre la valeur limite et la concentration des sols d'or, *Rec. trav. chim. Pays-Bas*, 39 : 613–617 (1920).

[3] De Jong, H. G. B., Contribution to the Theory of Vegetable Tanning I; Dehydration of Lyophylic Sols and Gels by Tannins and Its Bearing on the Theory of Vegetable Tanning, *Rec. trav. chim. Pays-Bas*, 42 : 437–472 (1923).

[4] De Jong, H. G. B., Contributions to the Theory of Vegetable Tanning II. Dehydration of the Gelatine Sol by Tannic Acid, Crystalline Tannins and Simpler Phenols, *Rec. trav. chim. Pays-Bas*, 43 : 35–67 (1924).

sols are stabilized not only by an electric charge but likewise by solvation. Figure 66 represents schematically de Jong's argument. In (*A*) we have a neutral particle. This can become negatively charged, as in (*B*), by the adsorption of an anion. The addition of a cation will reduce the charge to zero, and the particle will revert to its former condition as at (*C*). The adsorption of a cation will produce a positively charged micelle at (*D*), which again can be made isoelectric by the neutralizing effect of an electrolyte producing a particle, as at (*E*); or the particle (*A*) can become solvated (or hydrated if the dispersions medium is water) as at (*J*), and this solvated particle can, under the same conditions as above, become negatively charged, be neutralized, become positively charged, and again be neutralized without losing its liquid of solvation.

The addition of small amounts of electrolytes influences only the electrokinetic charge on the micelle. The addition of larger quantities of electrolytes influences the degree of solvation and causes flocculation to take place. In Fig. 66 flocculation will occur at (*A*), (*C*), and (*E*). (*B*) and (*D*) will form stable lyophobic sols, (*J*), (*H*), and (*F*) isoelectric lyophilic sols possessing a considerable degree of stability, and (*I*) and (*G*) charged lyophilic sols having a high degree of stability. Dehydrating agents, such as alcohol, will cause the lyophilic colloid to lose its liquid of solvation and accordingly effect the transformation from (*I*) to (*B*) or from (*G*) to (*D*). Because of the influence which the liquid of solvation has upon the stability of a lyophilic system, the apparent effect of electrolytes on such systems is much less marked than in the case of lyophobic sols.

The " salting out " of lyophilic colloids, such as proteins, from solution, by the addition of ammonium sulfate or by saturating the solution with magnesium sulfate is not an electrokinetic phenomenon whereby the electric charge is decreased to zero, but rather is a dehydration of the neutral micelle whose electrokinetic potential has already been reduced to nearly zero by the first addition of the electrolyte but whose affinity for the solvent is overcome only in solutions which contain a large amount of the electrolyte where the ions of the electrolyte compete with the lyophilic colloid for the water which is available.

Protective Colloids.—Inasmuch as lyophilic colloids are stabilized not only by an electric charge but by the adsorbed dispersions medium, small amounts of lyophilic colloids are, in many instances, sufficient to "protect" lyophobic sols from the flocculating action of electrolytes. This behavior is known as *protective colloid action.*

Schulz and Zsigmondy[5] have given us a measure of the protective

[5] Schulz, F. N., and Zsigmondy, R., Die Goldzahl und ihre Verwertbarkeit zur Charakterisierung von Eiweissstoffen, *Beitr. chem. physiol. Path.*, 3 : 137–160 (1903).

efficiency of lyophilic colloids in the "*gold number.*" They define the gold number of a colloid as *that weight of the colloid in milligrams which will just fail to prevent a change, in color from red to violet when* 1 *cc. of* 10 *per cent sodium chloride solution is added to* 10 *cc. of a Zsigmondy (formaldehyde) red gold sol to which the colloid has been added.* In all probability the mechanism of the reaction is due to an adsorption of the protective colloid on the surface of the gold micelles, so that the interface is no longer a gold-water interface but rather a hydrophilic colloid-water interface, the micelle acquiring the characteristics of the lyophilic colloid which was added. Zsigmondy has presented rather definite evidence that this is the case. If a sheet of gold foil is immersed in a gelatin solution, gelatin is adsorbed onto the interface and the gold will no longer amalgamate with mercury even after the gold has been washed in a stream of hot water. Zsigmondy explains the lack of amalgamation as due to the formation of a surface film of gelatin which prevents the mercury from getting in contact with the metallic gold. Similarly, the micelles in gold sols which have been protected with lyophilic colloids cannot be made to amalgamate with mercury. Table XXVII shows the gold numbers of various lyophilic colloids.

TABLE XXVII

Gold Numbers of Various Lyophilic Colloids

Substance	Gold Number	Substance	Gold Number
Dextrin (British gum)...	125–150	Gum arabic.....	0.10 –0.125
Soluble starch.....	10–15	Protalbinic acid.....	0.15 –0.20
Sodium oleate.....	2–4	Lysalbinic acid.....	0.10 –0.125
Egg albumin.....	0.08–0.10	Gelatin.....	0.005–0.0125

Colloidal silver and colloidal silver oxide have pronounced bactericidal properties, and the " argyrol " of the physician is colloidal silver protected by protein split-products, usually " protalbinic " or " lysalbinic " acid. The terms, " protalbinic " and " lysalbinic " acids, probably do not represent pure chemical compounds but rather mixtures of peptones prepared by partial hydrolysis of proteins (cf. Kennedy and Gortner[6]).

The protective action is characterized by a definite time interval. Sufficient time must elapse after the protective colloid has been added to the red gold sol to complete the initial adsorption of the protective colloid at the gold-water interface. Otherwise erroneous values will be obtained. The time interval before the sodium chloride is added, as a

[6] Kennedy, Cornelia, and Gortner, R. A., The Nitrogen Distribution in Protalbinic and Lysalbinic Acids, *J. Am. Chem. Soc.*, 39 : 2734–2736 (1917).

rule, need not exceed ten minutes. If the protective colloid is added to the solution of the electrolyte and then this mixture added to the colloid system, very much smaller quantities of electrolyte are sufficient to coagulate the lyophobic sol than would have been the case if the protective colloid had first been added to the sol. The explanation probably lies in the fact that the colloidal micelles adsorb the protective colloid and form a micelle which is not readily flocculated. No such pronounced adsorption takes place between the solution of the electrolyte and the lyophilic colloid, and when this mixture is added to the lyophobic system, sufficient time does not elapse for the stabilizing adsorption to take place, flocculation occurring at once.

Recently the gold number has been introduced into medicine, the protective value of the cerebrospinal fluid being used as an aid in the diagnosis of certain forms of insanity. The constituents of the cerebrospinal fluid which are responsible for the differences which are noted between normal and pathological samples have not as yet been identified.

Various other measures of protective value have been suggested. Thus, Windisch and Bermann[7] have proposed an *iron number* to characterize hydrophilic colloids which prevent the coagulation of Fe_2O_3 sols. They applied this technic to various colloid mixtures, obtained from beer, and which it was impossible to distinguish from each other by ordinary analytical procedure, in order to determine what colloids were responsible for the foaming power of beer. The various colloids in beer were separated by ultrafiltration, and the iron number of the fraction which caused foaming was then compared with the iron numbers of various constituents of malt or wort. They identified a gum in the wort, by this procedure, as being responsible for stabilizing the foam of beer.

Wo. Ostwald[8] has suggested a *rubin number*, in which Congo red sols are used in place of the gold sol, the rubin number being defined as the amount of colloid in grams per 100 cc. of solution which prevents the change of color of a Congo red sol from red to blue. The electrolyte used is potassium chloride, 160 millimolar concentration.

Undoubtedly various hydrophilic colloids will give different values for the iron number, the gold number, and the rubin number, and within a group of hydrophilic colloids the various values for these numbers will not necessarily follow the same order. Only a few of the factors involved

[7] Windisch, W., and Bermann, V., Über die Bedingungen für das Schaumen der Bierwürze, *Wochenschr. f. Brauerei*, 37 : 129–132 (1920).

[8] Ostwald, Wo., Ueber "Schutzwirkungen" beim Elektrolyt-Umschlag des Kongorubins und über die "Rubinzahl" einiger organischer Kolloide, *Kolloidchem. Beihefte*, 10 : 234–243 (1919).

in protective action have been ascertained in a qualitative way, and as long as different lyophobic micelles possess different electrokinetic potentials and different interfacial tensions, they must possess different adsorptive capacities, and the molecules which are adsorbed are probably not always oriented in a similar manner. Due to these variations, it is probably too much to expect that gold numbers should exactly parallel the iron numbers, or that either one of these should parallel the rubin numbers.

If the electrokinetic charge on the hydrophobic micelle is opposite

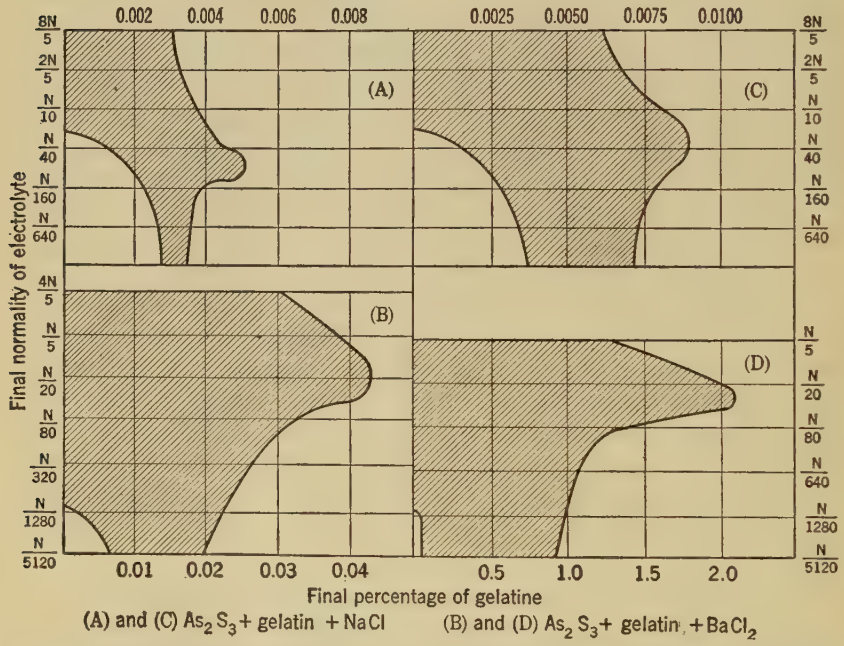


FIG. 67.—Showing sensitization and protection of As_2S_3 sols by gelatin. Complete flocculation occurs in the shaded areas. (Data of Sugden and Williams.)

in sign to the charge on the hydrophilic colloid which is being adsorbed, protection is not conferred by small additions of the hydrophilic colloid, but rather the system is made very unstable, due to a neutralization of the charge on the lyophobic micelle by the charge carried by the hydrophilic micelle.

The sequence of reactions is (1) increased sensitivity toward electrolytes, (2) neutralization of the charge, with the resulting flocculation, and (3) if sufficient amount of the hydrophilic colloid is added, the reversal of the charge on the lyophobic micelle, and protection.

Figures 67 and 68, taken from the work of Sugden and Williams,[9] show such initial sensitization and ultimate protection of negatively charged As_2S_3 and positively charged $Al(OH)_3$ sols by gelatin. The shaded areas in these figures denote the zones of complete flocculation.

Wright and Kermack[10] have made a similar study, using colloidal gum benzoin and hemoglobin sols at varying hydrogen ion concentrations and varying concentrations of gelatin. Figures 69 and 70 show the results that they obtained.

In a subsequent paper, they[11] utilized the technic developed in their study with gum benzoin and hemoglobin to test the properties of normal cerebrospinal fluid and cerebrospinal fluid from patients suffering with general paralysis of the insane. Figure 71 shows the areas of complete precipitation for normal cerebrospinal fluid, and Fig. 72, the results obtained with cerebrospinal fluid from the pathological cases. In Fig. 72 the area of precipitation shown by the vertical lines is the area characteristic of normal cerebrospinal fluid. The area denoted by the horizontal lines appears to be characteristic of cerebrospinal fluid from cases of general paralysis of the insane, and Wright and Kermack note that the constituents which cause flocculation of gum benzoin in this area are abnormal constituents not present in the cerebrospinal fluid of normal persons.

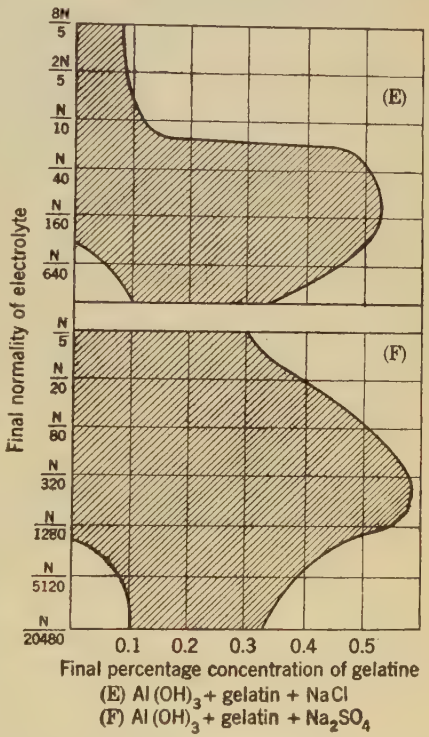


FIG. 68.—Showing sensitization and protection of $Al(OH)_3$ sols by gelatin. Complete flocculation occurs in the shaded area. (Data of Sugden and Williams.)

[9] Sugden, S., and Williams, M., An Experimental Study of Protective Colloids. Part I. The Influence of Concentration, *J. Chem. Soc.*, London, pp. 2424–2432 (1926).

[10] Wright, H. D., and Kermack, W. O., The Properties of Colloidal Gum Benzoin, *Biochem. J.*, 17 : 635–657 (1923).

[11] Wright, H. D., and Kermack, W. O., The Mechanism of Precipitation of Colloidal Gum Benzoin by Cerebrospinal Fluid, *Biochem. J.*, 17 : 658–667 (1923).

Mutual Precipitation of Colloids.—The preceding paragraphs have indicated that positively charged colloids will cause flocculation when added to systems containing negatively charged micelles, and *vice*

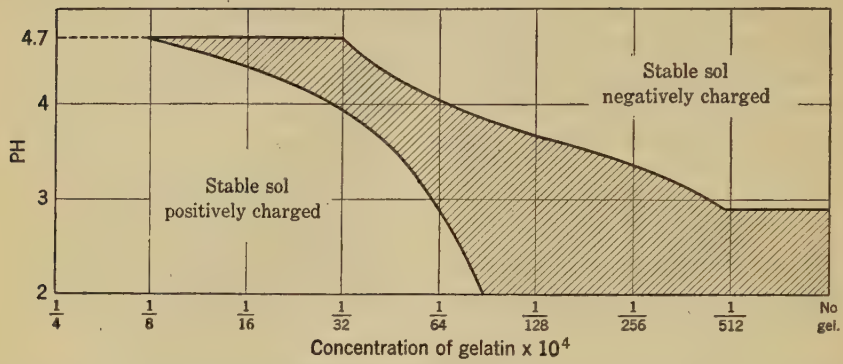


Fig. 69.—Showing the areas characteristic of the flocculation of gum benzoin sols by gelatin sols at various hydrogen ion concentrations. (Data of Wright and Kermack.)

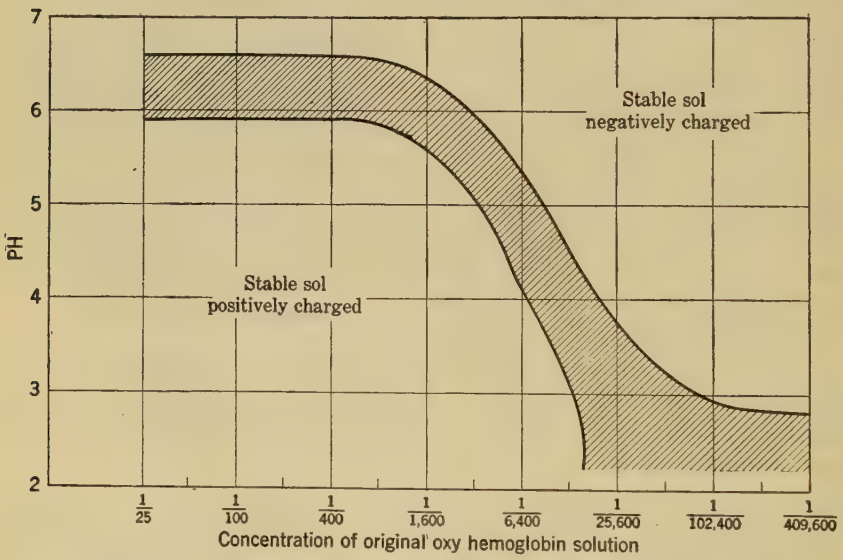


Fig. 70.—Showing the areas characteristic of the flocculation of gum benzoin sols by hemoglobin sols at various hydrogen ion concentrations. (Data of Wright and Kermack.)

versa. This property can often be utilized for the qualitative determination of the sign of the charge on the micelles in a given system. Filter paper (cellulose) is negatively charged against water, and if a strip of

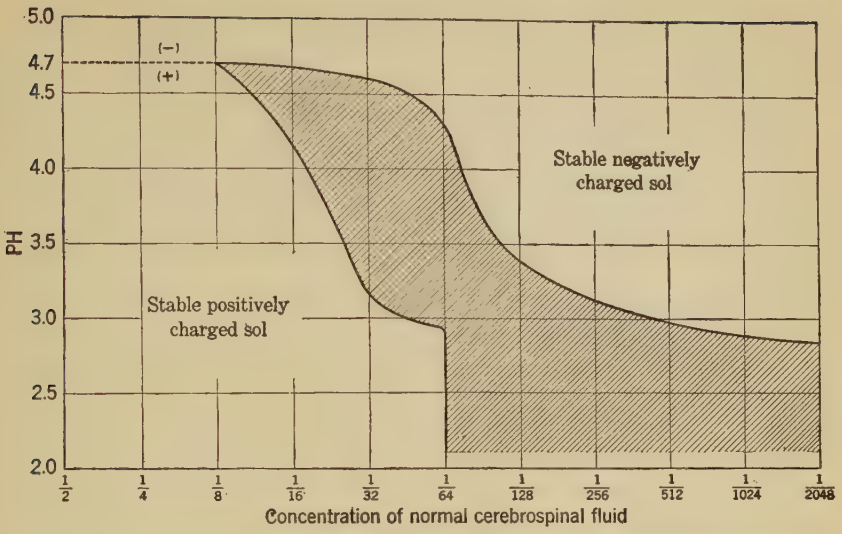


FIG. 71.—Showing the areas characteristic of the flocculation of gum benzoin sols by normal cerebrospinal fluid. (Data of Wright and Kermack.)

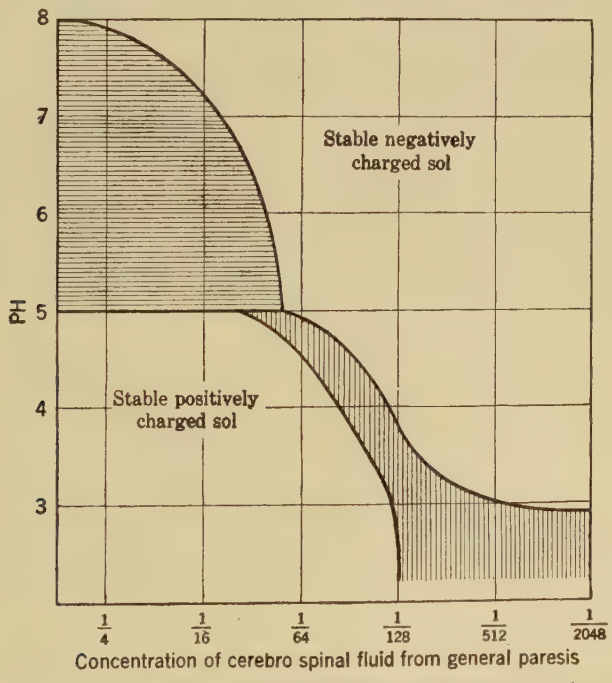


FIG. 72.—Showing the areas characteristic of flocculation of gum benzoin sols by cerebrospinal fluid from pathological cases. (Data of Wright and Kermack.)

filter paper be dipped into a hydrosol, the liquid will be drawn up by capillary action. If the micelles present in the sol are negatively charged, they will ascend the strip of filter paper, although as a rule at a somewhat slower rate than the water. On the other hand, if the micelles are positively charged, their charge will be neutralized at the point where the filter paper touches the surface of the sol and only the dispersions medium will ascend by the capillary action. The reverse behavior would take place, providing the absorbing column were a positively charged colloid.

This method of determining the sign of the charge on colloid micelles is known as *capillary analysis*. Capillary analysis can sometimes be advantageously applied to determine whether or not a chemical system contains only one kind of colloidal micelles. Thus, for example, if a purple dye solution is tested by capillary analysis, it may be found that the filter paper is colored a uniform purple color, in which case the dyestuff present is probably a single chemical compound, or the filter paper may have a zone of red (or blue) advancing ahead of the purple boundary, in which case the purple solution was prepared by mixing red and blue dyestuffs. The purity of a dye can, as a rule, be roughly ascertained by this method, due to a difference in rate of diffusion between mixtures of various dyestuffs.

The mutual precipitation of colloids is of extreme importance in the industry. Dyeing of fabrics is largely dependent upon the adsorption of the dye by the fiber. Animal fibers, such as wool, are amphoteric, *i.e.*, the charge on the fiber is rather readily altered so as to be either positive or negative, depending on the hydrogen ion concentration of the medium. Animal fibers, therefore, can be dyed with either positive or negative dyestuffs by simply shifting the hydrogen ion concentration of the dye bath. As a rule, however, the positively charged basic dyestuffs are preferable for dyeing animal fibers.

Cotton is characterized by possessing a negative charge which cannot be readily reversed by changes in hydrogen ion concentration. Certain dyestuffs, known as substantive dyes, color cotton directly. These substantive dyes, derivatives of the benzidine series, possess basic groups, and the micelles carry a positive charge. The attraction between dyestuff and fiber is, therefore, the attraction between positively charged and negatively charged colloids. Mutual precipitation occurs on or in the interstices of the fiber, and the adsorbed dyestuff is firmly fixed on the oppositely charged fiber.

In order to dye cotton or other negatively charged fibers with acid dyestuffs, it is necessary to mordant the fiber. The mordants commonly used are aluminum or chromium salts. The fiber is put through a mor-

danting bath where Al^{+++} or Cr^{+++} is adsorbed by the negatively charged fiber in such amounts that the original negative charge on the fiber is reversed by the positively charged metallic ions, the mordanted fiber acquiring a strong positive charge which permits of the adsorption and mutual precipitation of the negatively charged dyestuff on the surface and in the interstices of the fiber.

Moore[12, 13] has utilized the principle of the mutual precipitation of colloids in order to prepare arsenical insecticides which will adhere over long periods to the foliage of plants. Arguing from the fact that plant tissues in general possess a negative charge, Moore reasoned that the particles of a positively charged insecticide would be drawn underneath the water film which covers all leaf surfaces and would be so firmly adsorbed by the leaf that they would not be washed off by the rain. He believed that the reason that the usual arsenical preparations are so readily washed off by rains might be that the insecticide particle never came in contact with the surface of the leaf because of the opposing forces of two negatively charged surfaces.

Diagram (*A*), Fig. 73 shows the hypothetical picture of a positively charged particle neutralized by being adsorbed on a negatively charged surface, and the position which the water film would assume. Diagram (*B*), Fig. 73, shows the hypothetical position which would be taken by a negatively charged particle sprayed onto a negatively charged surface, with the relative position of the water film. It is easily seen that the particle in (*B*) would be readily washed off of the surface by a stream of water. Accordingly Moore determined by electroendosmotic technic the sign of the charge on the surface of leaves and found, as expected, that the leaves were negatively charged. He then tested by cataphoresis the available arsenical preparations and found that they were invariably negatively charged. Utilizing the principles involved in the complex theory of colloids, he argued that a lead arsenate carrying an excess of lead, or that a ferric arsenate carrying an excess of iron, should be positively charged, and he therefore prepared considerable quantities of such positively charged arsenicals.

FIG. 73.—A diagrammatic representation of the relationships which exist between a leaf surface with its adsorbed water film and a particle of (A) a positively charged or (B) a negatively charged insecticide.

[12] Moore, W., Spreading and Adherence of Arsenical Sprays, Minn. Agr. Exp. Sta., Technical Bull. No. 2, 50 pp. (1921).

[13] Moore, W., Adherent Arsenical Preparations, *Ind. Eng. Chem.*, 17 : 465–466 (1925).

Laboratory tests of adherence on leaf surfaces proved very striking. If leaves were sprayed with the negatively charged arsenical and, after drying, were placed under a spray of water, practically all of the arsenical was removed within a few minutes. On the other hand, if leaves were sprayed with the positively charged preparations, and then, while still wet, were immediately placed under a spray, a very considerable amount of the arsenic remained on the leaves after several hours' washing. Commercial negatively charged lead arsenate sprayed on plants in the field was still present on the leaves to the extent of only 6.6 per cent at the end of 17 days during which 3.07 inches of rain had fallen. Lead arsenate, to which a positively charged colloid, ferric hydroxide, had been added, adhered under identical conditions to the extent of 17.5 per cent.

In a personal communication to the author, Professor Moore gave the following results for a more extensive field trial. The positively charged arsenical was applied in a spray to the leaves of trees in the spring and when the leaves fell in the autumn after approximately 30 inches of rainfall, more than 20 per cent of the applied arsenic was found to be still adhering to the fallen leaves. It would accordingly appear as if positively charged insecticides and fungicides should be used for controlling insect pests and the fungus diseases. There would, of course, be a limit to their use, providing they were applied when fruit was on the tree, for by so doing the amount of arsenic adhering to the ripened fruit might introduce a dangerous factor.

Hooker[14] has applied this identical principle to the preparation of a copper fungicide. He finds that colloidal copper hydroxide, due to its positive charge, is an efficient fungicide when sprayed in a concentration of one part of the colloid to 5000 parts of water. The adherence was exceptionally good and the control of fungus diseases was equal to the control which was obtained with a 3 : 4 : 50 Bordeaux mixture, although the positively charged colloidal copper hydroxide spray contained only 6.8 per cent of the copper which was present in the Bordeaux mixture.

The Lyotropic Series.—Valency is not the only factor involved in the behavior of ions toward a colloid system. Thus, a group of monovalent ions possesses within itself varying degrees of ability to flocculate sols. Hofmeister was apparently the first one to investigate this phenomenon and in a series of papers[15-17] from his laboratory he showed the

[14] Hooker, H. D., Colloidal Copper Hydroxide as a Fungicide, *Ind. Eng. Chem.*, 15 : 1177–1178 (1923).

[15] Hofmeister, F., Zur Lehre von der Wirkung der Salze. Zweite Mittheilung, *Arch. exp. Path. u. Pharm.*, 24 : 247–260 (1888).

effects of various anions and cations upon protein systems. Numerous workers have since made similar studies, using both lyophilic and lyophobic systems, and it is generally agreed that the anions can be arranged in a series of citrate > tartrate > SO_4 > acetate > Cl > NO_3 > Br > I > CNS, where, at least for protein systems, citrate shows the greatest precipitating effect, and thiocyanate the least. The cation effects are somewhat less marked but may be expressed approximately as Th > Al > H > Ba > Sr > Ca > K > Na > Li.

These or similar series of ionic effects have come to be known as the lyotropic series, or the Hofmeister, or the irregular series of ions. Various theories have been propounded to account for the observed differences in the behavior of the various salts. The behavior of the lyotropic series of ions is not limited to its effect upon colloid systems, since it has been abundantly demonstrated in other connections. Thus, Jaeger[18] finds the surface tension of molten alkali salts at 1000° C. to show a series of F > SO_4 > Cl > Br > NO_3 > I and Li > Na > K > Rb > Cs, and essentially the same order holds for the effect of these ions in increasing the surface tension of water. Freundlich has suggested that in all probability the series correspond to the order of the hydration of the ions, the most hydrated ions being at the sulfate and lithium ends of the lyotropic series.

Frumkin[19] devised a technic for measuring the potential difference at an air-solution interface. Table XXVIII shows certain of his data. He accordingly constructs a series of ions, showing the effect of the anion on the phase-boundary potential of the order, F, SO_4 > Cl > Br > NO_3 > I > CNS, and suggests that this series is related to the hydration of the anions. A similar view has been expressed by Kruyt and Robinson[20] who suggest that molecular orientation may well be a factor. Kruyt and Robinson note that the solubility of various materials may be very different in a salt solution from what it is in pure water. In the case of quinone they found a lyotropic series of anions influencing solu-

[16] Lewith, S., Zur Lehre von der Wirkung der Salze, Erste Mittheilung, *Arch. exp. Path. u. Pharm.*, 24 : 1–16 (1888).

[17] Hofmeister, F., Zur Lehre von der Wirkung der Salze, Dritte Mittheilung, Fünfte Mittheilung, und Sechste Mittheilung, *Arch. exp. Path. u. Pharm.*, 25 : 1–30 (1889); 27 : 395–413 (1890); 28 : 210–238 (1891).

[18] Jaeger, F. M., Temperaturabhängigkeit der molekularen freien Oberfläschenenergie von Flüssigkeiten im Temperaturbereich von −80 bis +1650 C°., *Z. anorg. u. allgem. Chem.*, 101 : 1–214 (1917).

[19] Frumkin, A., Potentialdifferenzen zwischen Flüssigkeiten und Luft, *Koll. Z.*, 35 : 340–342 (1924); Phasengrenzkräfte und Adsorption an der Trennungsfläche Luft/Lösung anorganischer Elektrolyte, *Z. physik. Chem.*, 109 : 34–48 (1924).

[20] Kruyt, H. R., and Robinson, C., On Lyotropy, *Proc. Koninklijke Akademie van Wetenschappen*, Amsterdam, 29 : 1244–1250 (1926).

TABLE XXVIII

The Phase Boundary Potentials, ϵ, between Air and the Surface Layer of Certain Inorganic Salt Solutions (Data of Frumklin)

Electrolyte	Solution N/1, ϵ(Millivolts)	Solution 2 N ϵ(Millivolts)	Electrolyte	Solution N/1, ϵ(Millivolts)	Solution 2 N, ϵ(Millivolts)
KF	...	+ 5	Na_2CO_3	+ 3	+ 5
KCl	− 2	− 6	RbCl	..	− 7
KBr	−10	−16	CsCl	..	− 6
KI	−34	−52	LiCl	..	− 9.5
KCNS	−57	−87	$Al_2(SO_4)_3$	0	+ 6
KOH	0	+ 5	HF	−71	−103
K_2CrO_4	..	− 1	HCl	−23	− 55
K_2CO_3	..	+ 3	HBr	−34	− 78
NaI	−39	−55	HI	−61	−112
$NaNO_3$	−17	−23	H_2SO_4	−13.5	− 55(?)
NaCl	− 1	− 4	H_3PO_4	− 0.5	
Na_2SO_4	+ 3				

bility, the solubility of quinone in a 1.5 molar solution of KCNS being 170 per cent of its solubility in water, whereas in the case of K_2SO_4 of an equivalent concentration the solubility was only 63.7 per cent of the water solubility. When hydroquinone was used, it was found that the cations showed the more pronounced lyotropic series, ranging from CsCl solutions, where the solubility of the hydroquinone was but slightly lower than in pure water, to LiCl, where the solubility was only 56.6 per cent of the water solubility. Table XXIX lists certain of the data referred to.

TABLE XXIX

Showing the Relative Solubility of Quinone and Hydroquinone in Solutions of Various Inorganic Salts (Data of Kruyt and Robinson)

	Solubility as Percentage of Water Solubility				
	Quinone	Hydroquinone		Quinone	Hydroquinone
H_2O	100	100	1.5 M K_2SO_4	63.7	
1.5 M KCNS	170.1		1.5 M CsCl	97.6	95
1.5 M KI	149.6	74.8	1.5 M NaCl	80.3	59.1
1.5 M KNO_3	132.1	80.0	1.5 M LiCl	77.3	56.6
1.5 M KBr	108.5	70.8	1.5 M RbCl	93.8	
1.5 M KCl	90.4	67.0			

Somewhat similar lyotropic series were found by Kruyt and Robinson in the solubility of the various nitroanilines in aqueous solution. The explanation which they propose is that there is an orientation of the dipoles of water in the immediate neighborhood of the ions and that two kinds of orientation are possible. Either the positive end or the negative end of the water molecule may be turned toward the molecules of the solute. Similar orientations of water are postulated to occur on the anions and cations of the electrolytes, and it is the interaction of these three orientations that determines the behavior of a given system.

Briggs[21] has summarized the literature in regard to the effect of ions on electrokinetic potentials, and reaches the conclusion that the lyotropic series is dependent not only on ionic mobility and hydration, but also on a number of other factors, only a few of which at the present time can be quantitatively evaluated. Among these factors is the ζ-potential. Figure 74 shows changes in the electrokinetic potential which Briggs found for a series of chloride solutions against a cellulose membrane, as measured by streaming potential methods. Briggs explains the initial increase in the negative charge of a cellulose surface, when small amounts of the chlorides of Li, Na, K, and Cs are added, as being due to a greater adsorption of the anion than of the cation. At slightly higher concentrations a relatively

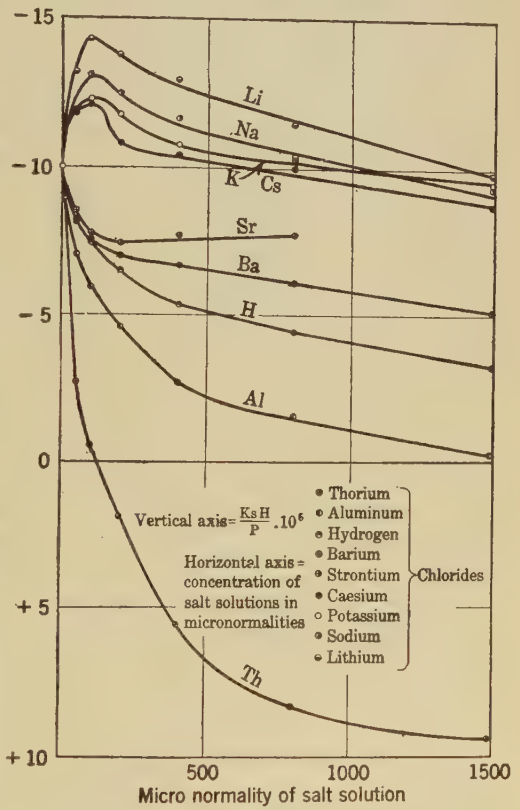


FIG. 74.—Showing a lyotropic series of ions as determined by measuring the electrokinetic potentials at a cellulose-salt solution interface. (Data of Briggs.)

[21] Briggs, D. R., The ζ-Potential and the Lyotropic Series, *J. Phys. Chem.*, 32 : 1646–1662 (1928).

greater adsorption of the cation takes place, and the charge is again decreased. The divalent ions, strontium and barium, are more strongly adsorbed throughout the entire range of concentrations than is the chloride ion. They are, however, not sufficiently adsorbed in the concentrations studied, to reduce the electrokinetic charge to zero. In these studies the thorium ion was the only ion which reversed the charge on the cellulose interface.

The mere fact that a reversal of the charge is possible and a high positive electrokinetic potential is built up at increasing concentrations of the thorium ion led Briggs to conclude that the electrical free energy at the interface is not the only form of free surface energy which is being acted upon by the adsorbed salt and in reality it may be only secondary to some other free surface energy change taking place at the same time. Thus, Briggs states, "At an interface between two phases there is always present a condition of strain, due to unsaturation of partial valence forces. A part of this free energy of the surface zone will usually be manifested by the presence of an electrical potential difference. This potential arises from the fact that, when a pair of ions, one plus, the other minus in charge, are brought into the interface region, one will nearly always show a greater tendency to lower the free energy of the surface layer than the other and will therefore be forced into that layer with more force than its mate. We can picture a condition in which one of a pair of ions would tend to lower the surface energy to a large extent, whereas its mate would, if brought into the surface layer, cause an increase in the surface energy. In such a case, the former would move into the surface layer, while the latter would not and, while the surface energy as a whole would be lowered, a large electrical potential difference would at the same time be formed. If both ions had about the same effect upon the surface free energy, either to decrease it or increase it, they would be equally attracted to or repelled from the surface layer and no change would occur in the electric potential present there.

"The magnitude of the ζ-potential at an interface, therefore, does not necessarily parallel in value the adsorption capacity of that interface. It is only one of the forms in which free surface energy may be present and is, therefore, no more important than is surface tension, or any other condition of strain in which the surface energy may be able to exist, in defining the free energy content of the interface phase of a system. In order to completely define the conditions in the interface, the magnitude of all forms in which surface energy can exist must be determined. Adsorption of some substance may take place to a large degree without appreciably altering the magnitude of some forms of free surface energy. For example, it is conceivable that the free energy as exhibited by inter-

facial tension may be lowered out of all proportion to any corresponding change in the free surface energy represented by the electrokinetic potential. Other substances may affect all forms of the energy when they are adsorbed. Some substances may be adsorbed to cause a decrease in the total free energy of the surface layer, but at the same time increase the amount of free surface energy existing in some particular form.

" In the case of thorium chloride, we may assume that the total free surface energy is decreased with increased concentrations. The electrical free energy of the surface layer, however, is first decreased to zero and then increased in the opposite direction, showing that the thorium ion had been strongly oriented toward the surface layer even after the charge had been reversed and, while the total free surface energy is being lowered with increased concentration, the electrical free surface energy is being increased. At still higher concentrations of thorium chloride, it has been found that the ζ-potential is lowered toward zero again by the increased effect that the Cl^- ion has at these higher concentrations."

Briggs is undoubtedly correct in arguing that the behavior of any chemical ion will depend not only upon the concentration of that ion but also upon the nature of the system and upon the type of reaction which is being studied. This explains why one author will give a certain lyotropic series as illustrative of the effect of ions on viscosity, and another worker will give a very similar but somewhat different series for the effect of the same ions in flocculating a sol or in causing peptization. Probably each worker was securing results which indicated the behavior of ions in the system which he was studying, but such behavior would be slightly altered if other and different factors were introduced.

Gortner, Hoffman, and Sinclair[22, 23] have presented a rather extensive study of the peptizing behavior of various inorganic salts on wheat flour proteins and find, as was expected, that there is a very pronounced lyotropic series. Perhaps the most striking series is represented by the potassium halides, where in 1.0 N solution KF peptized on the average 13.07, KCl 22.77, KBr 37.22, and KI 63.89 per cent of the wheat flour proteins. The bearing of this work on the subject of protein chemistry will be discussed later, it being sufficient to point out here that *within a series of solutions of equal ionic concentration, there will be found very marked differences in the behavior of such solutions toward protein systems.*

[22] Gortner, R. A., Hoffman, W. F., and Sinclair, W. B., Physico-Chemical Studies on Proteins III. Proteins and the Lyotropic Series, Colloid Symposium Monograph, Vol. V, pp. 179–198 (1928).

[23] Gortner, R. A., Hoffman, W. F., and Sinclair, W. B., Zur Kenntnis der Proteine und der lyotropen Reihen, *Koll. Z.*, 44 : 97–108 (1928).

The removal of an ion from an interface may be often brought about by adding another ion of the system. Thus, Seth[24] has pointed out that adsorption is the predominant feature in the interaction between the accumulative poisons, such as lead, arsenic, copper, and mercury, and proteins of the body. The heavy metals are selectively adsorbed by the body proteins and tend to alter the nature of the proteins until finally sufficient quantities are accumulated to cause death.

Carder and Coffindaffer[25] argued that if mercury were adsorbed by the proteins, it should be possible to replace mercury by using an innocuous electrolyte. Accordingly they set up an experiment in which 38 dogs were given a fatal dose of 20 mg. $HgCl_2$ per kilo of body weight. Fifteen of these dogs received no further treatment and all died from mercury poisoning within 1 to 6 days. The 23 remaining dogs received periodically intravenous and intraperitoneal injections of an 0.8 per cent solution of NaCl which were repeated daily until either death or apparent recovery occurred, one month being selected as the time limit. Nineteen of the 23 dogs receiving the injections of NaCl survived. We are here dealing with the replacement of a readily adsorbed metal by ions which, while they are only slightly adsorbed, are present in such a high concentration as to render possible the removal of the mercury from the interface, illustrating the effect which even a monovalent ion may have on the colloidal behavior of an ion of higher valence.

Electrical Precipitation.—A discussion of the precipitation of suspended particles upon electrified surfaces can be justified at this point because the same basic principles hold for such precipitation as for the precipitation by means of electrolytes.

Cottrell[26] has discussed the general theory which is involved and has devised apparatus for satisfactorily causing the precipitation of suspended particles. He notes that, " If we bring a needle point connected to one side of a high potential direct current line opposite to a flat plate connected to the other side of the line we find that the air space between becomes highly charged with electricity of the same sign as the needle point irrespective of whether this is positive or negative, and any insulated body brought into this space instantly receives a charge of the same sign. If this body is free to move, as in the case of a floating particle, it will be attracted to the plate of opposite charge and will move

[24] Seth, Trilok Nath, Adsorption and Mechanism of Poisoning, Pt. I. Irritant Poisons, *Biochem. J.* 17 : 613–621 (1923).

[25] Carder, J. R., and Coffindaffer, R. S., The Value of Forcing Fluid in the Treatment of Mercuric Chloride Poisoning, *J. Am. Med. Assoc.*, 81 : 448–450 (1923).

[26] Cottrell, F. G., The Electrical Precipitation of Suspended Particles, *J. Ind. Eng. Chem.*, 3 : 542–550 (1911); cf. also *J. Ind. Eng. Chem.*, 11 : 147–153 (1919).

at a rate proportional to its charge and the potential gradient between the point and plate."

Such a method of precipitation has come to be known as the Cottrell precipitation. A high tension current is applied to chains or rods hung in the stack and the particles suspended in the gas, becoming electrified, are later precipitated upon plates having the opposite electrical charge, in the same way that two oppositely charged colloidal particles would attract and neutralize each other. The method has been applied not only to gases from smelters but likewise to the dusts from cement plants where Schmidt[27] notes that in a single cement plant at Riverside, California, approximately 350,000 tons of dust have been collected by the use of the Cottrell process during a twelve-year period. In this particular installation the treatment was necessary because of the fact that the cement dust, settling on the citrus groves, interfered with normal photosynthesis. Similar installation has also been used in the breaking of emulsions, particularly natural emulsions of crude petroleum and salt water, as noted by Sherrick,[28] and Speed.[29]

Recently McClendon[30] has constructed a laboratory precipitator in order to avoid the loss of dust when relatively large quantities of grains or other foodstuffs are burned in the process of analyzing them for iodine content. Although the gases of combustion were passed several times through solutions, McClendon observed that solid particles were still present in the gases, and feeling that iodine might be lost, he passed the gases through a Cottrell precipitator. Even at a high velocity of gas flow, he was able to precipitate the suspended particles practically quantitatively. Undoubtedly there are many occasions in the laboratory where similar technic could be employed advantageously.

[27] Schmidt, W. A., Electrical Precipitation in Retrospect, *Ind. Eng. Chem.*, 16 : 1038–1041 (1924).

[28] Sherrick, J. L., Oil-Field Emulsions, *J. Ind. Eng. Chem.*, 12 : 133–139 (1920).

[29] Speed, B., An Appreciation of Dr. Cottrell, *J. Ind. Eng. Chem.*, 11 : 153–154 (1919).

[30] McClendon, J. F., The Determination of Traces of Iodine. I. *J. Am. Chem. Soc.*, 50 : 1093–1099 (1928).

CHAPTER VIII

GELS

Gels may be defined as more or less rigid colloid systems. In many instances they differ from lyophilic sols only in concentration or in degree of dispersion, *i.e.*, a sol may in some instances be transformed into a gel by increasing the content of the disperse phase or may spontaneously pass into a gel by an aggregation of the micelles. Conversely a gel may pass into a sol by the mere process of the peptization of the disperse phase. In the true gels, characteristic of the lyophilic colloids, the gel $\leftrightharpoons$ sol transformation is more or less readily reversed. Thus, for example, with gelatin, temperature and hydrogen ion concentration in a large measure determine whether a given system will be a sol or a gel. Similarly, a starch-water suspension may be converted into a true sol at an elevated temperature and the system will set to a gel when the temperature is lowered.

Coagula produced by the addition of electrolytes to lyophobic systems are often referred to as gels. Such coagula, however, cannot be re-converted into sols without rather drastic peptization treatment.

The apparent viscosity or plasticity of gels may vary from a very thin " jelly," which will flow under rather slight pressure, to rigid structures, such as wool or cotton fibers, filter paper, horn, hair, etc., and the properties of the systems will vary with their physical structure.

Undoubtedly gels have a structure. Two general views have been put forward as to the structure of gels. Bütschli maintained that gels have a more or less honey-comb structure, the disperse phase forming a net-work of cell-like walls, the interior being filled with the dispersions medium. Nägeli, on the other hand, suggests a brush-heap structure, in which minute ultramicroscopic fibrils are interlaced throughout the system and the dispersions medium is held within the fibrillar mass by capillary forces in the same way that a crystalline mass of tyrosine or caffeine possesses a certain degree of rigidity.

The evidence appears to be entirely in favor of the brush-heap structure. The only instance of which the author is aware in which a non-lyophilic substance readily forms a gel is dibenzoyl-*l*-cystine, as noted by

Gortner and Hoffman.[1] Using this material, they were able to prepare relatively rigid gels which contained only a small amount of the disperse phase. As little as 0.2 per cent of dibenzoyl-*l*-cystine formed a relatively rigid hydrogel and even 0.1 per cent formed a soft gel. Ultramicroscopic studies indicated that these gels were of a brush-heap structure, formed by the interlacing of relatively long crystals of dibenzoyl-*l*-cystine, the crystals being so thin as to have no apparent cross section. Dibenzoyl-*l*-cystine is not hydrophilic and does not crystallize with water of crystallization. The extreme minuteness of the crystal fibrils apparently formed an intricate net-work immeshing the dispersions medium. In spite of the fact that dibenzoyl-*l*-cystine does not have associated with it water of crystallization, it must nevertheless be regarded as a polar substance, due to the presence of amino groups, carboxyl groups, and the —S—S— linkage. We would accordingly expect it to attract water, a polar liquid.

Kraemer[2] has used the motion picture camera as an adjunct to ultramicroscopic studies and has studied gelatin gels and dibenzoyl cystine gel during the gelling process. The gelatin gels were also studied during the melting process. By introducing small mercury particles into the gels, he was able to show that the resistance of the gel to Brownian movement was not uniform, but that in the case of a dibenzoyl cystine gel the space between the fibrils was occupied by the dispersions medium which appeared to have essentially the same viscosity as water in bulk. In the case of gelatin gels, channels appeared to exist where the Brownian movement of the mercury particles was but slightly impeded, and at other points definite resistance to Brownian movement could be demonstrated. Although no fibers were visible at these points of apparent resistance to the Brownian movement of the mercury particle, it appears as if fibers having approximately the same refractive index as water, may well have been present in these areas.

Furthermore, if Bütschli's view were correct, one would expect to find a resistance to the diffusion of a given material or the resistance to the passage of an electrical current through a dilute gel. Such differences, however, if they do exist, are extremely slight.

The author has observed many instances where a plant sap, when expressed from leaves, was, while relatively viscous, definitely not a gel. The specific electrical conductivity of such a system remained constant during and following complete gelation, and in a number of instances the

[1] Gortner, R. A., and Hoffman, W. F., An Interesting Colloid Gel., *J. Am. Chem. Soc.*, 43 : 2199–2202 (1921).

[2] Kraemer, E. O., Studies with the Kinoultramicroscope, Colloid Symposium Monograph, Vol. II, pp. 57–69, Chemical Catalog Company, Inc., New York (1925).

gel structure underwent contraction with the squeezing out of a clear fluid and the separation of a distinct " clot " of organic material, the liquid which was squeezed out of the clot being perfectly limpid and still possessing the same specific conductivity as was possessed by the freshly expressed sap or by the gel. It is only when a system possesses a relatively high percentage of disperse phase that the specific electrical conductivity decreases even slightly on passing from a sol to a gel.

Many of the earlier papers on gel structure were based on observations of sections of gels which had been studied by the usual microscopic technic, *i.e.*, the gels had been " fixed " and hardened by the usual biological laboratory reagents, following which they were dehydrated, sectioned, and studied under the microscope. It should be pointed out here that the fixing and hardening reagents of the cytological laboratory are in reality reagents which transform the hydrophilic biological colloids into lyophobic systems. Accordingly one of the most important phases of the bio-colloid system, namely the water, is eliminated at an early stage in the process, and undoubtedly rearrangements take place between the organic portions when their affinity for water is destroyed. It may well be that the distribution of the organic colloid particles is entirely different in the original gel from what it appears to be in the fixed, dehydrated, and stained sections which are ultimately studied. The honey-comb structures originally observed by Bütschli appear to have been artifacts.

The tendency of a given system to gelate is in direct proportion to the affinities which exist between the disperse phase and the dispersions medium. If the dispersions medium is strongly attracted by the disperse phase, so that the disperse phase becomes highly solvated, the particles of the disperse phase become surrounded by " shells " of the dispersions medium, the apparent viscosity of the system is greatly increased, until finally the system becomes more or less rigid and a gel results. We are dealing in such instances with surface forces where electrical attractions, interfacial tension forces, and molecular orientation are all operative. In many instances it is impossible, with our present knowledge, to determine exactly what forces are responsible for the phenomena observed in a given system.

The Formation of Gels.—The general methods for the formation of gels may be divided into four groups.

1. The Allowing of a Sol to " Set " after a Change in Temperature, Hydrogen Ion Concentration, Electrolyte Content, etc.—Typical examples are the formation of a gelatin gel from a gelatin sol by the lowering of the temperature, or the formation of a silicic acid gel by the addition of an acid to sodium silicate. In the case of gelatin a considerable period

of time must elapse before a rigid gel is obtained. In the case of sodium silicate the gel forms within a relatively few minutes.

2. **The Dialyzing Out of a Peptizing Agent from a Sol.**—If a strong solution of ferric chloride is added to an equivalent solution of sodium arsenate or of phosphoric acid, clear, limpid sols will result. If these sols are dialyzed until the excess electrolytes have been removed, gels will be obtained.

3. **The Solid Imbibing the Dispersions Medium.**—This is the simplest method of gel formation and is typified by the swelling of gelatin in water, the swelling of rubber in organic solvents, and is characteristic of most of the bio-colloids.

4. **The Addition of a Third Phase to a System.**—Cellulose nitrate, for example, is not appreciably peptized by either ethyl alcohol or by ethyl ether, but is readily peptized by a mixture of the two. In some instances the addition of a small amount of a third material may produce relatively great changes in a given system. Thus, for example, Holmes and Maxson[3] found that traces of water in systems of turpentine and sodium stearate, potassium stearate, and sodium oleate had a very great effect upon the gelating properties of such systems, in the case of the sodium stearate favoring the formation of gels, and in the case of sodium oleate inhibiting gel forma-

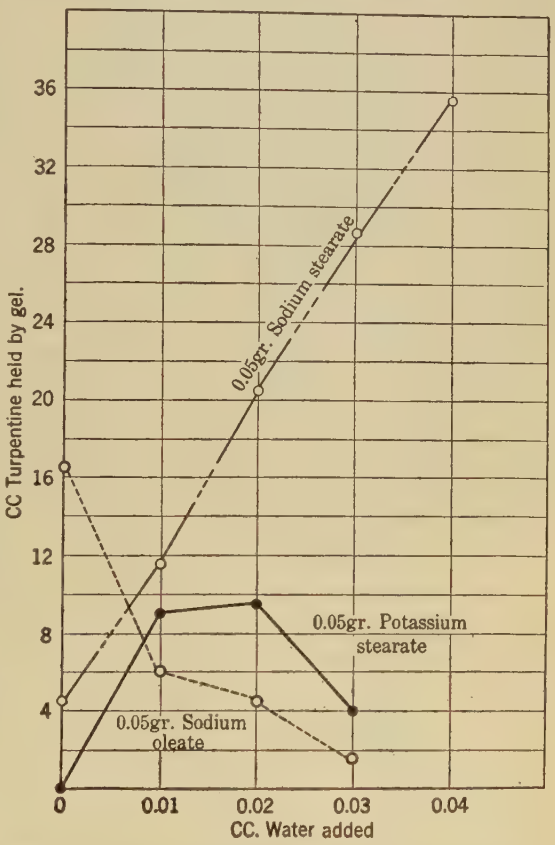


FIG. 75.—Showing the influence of minute traces of water on the formation of turpentine-soap gels. (Data of Holmes and Maxson.)

[3] Holmes, H. N., and Maxson, R. N., The Influence of a Second Liquid upon the Formation of Soap Gels, Colloid Symposium Monograph, Vol. V, pp. 287–300 (1928).

tion. Figure 75 shows the maximum amount of turpentine which would be taken up and held in the form of a gel by 0.05 gm. of the disperse phase in the presence of varying amounts of water. No satisfactory explanation for the observed behavior of the above system has been advanced.

The Solvation of Lyophilic Colloids.—The lyophilic colloids are distinguished from the lyophobic colloids by an affinity for the dispersions medium. Whether the micelles of the disperse phase actually act as a solvent for the dispersions medium or whether the dispersions medium is simply oriented in a "shell" surrounding the disperse phase is still an open question, although the recent evidence tends to support the latter viewpoint. The rapidity with which the dispersions medium may be "bound" and set free is so great that it seems hardly probable that the relatively slow process of diffusion can be a factor, as it would be if molecules of the dispersions medium actually penetrated into the interior of the micelles. Solvation is easily followed by changes in viscosity. In Fig. 13 is shown the very rapid fall in viscosity which takes place in an acidulated flour-water suspension when traces of inorganic salts are added. In the particular experiments in question, the maximum viscosity reached yielded a fairly coherent gel and within, at the most, two or three seconds after the addition of the magnesium sulfate, the viscosity had fallen to practically that of the dispersions medium. It would appear as if a large fraction of the dispersions medium had simply been "bound" on the surface of the protein micelles, probably by electrical forces, and that this bound water had been released by the neutralizing effect of the inorganic salt.

Fig. 76.—Showing the relative size of colloidal particles of the dyestuff, "Geranin G," and the associated water shell. (Data of Liepatoff.)

Liepatoff[4] has attempted to estimate the thickness of the water shell which was adsorbed onto the surface of a colloidal particle of the dyestuff, Geranin G. He estimated the thickness by the change in viscosity of known concentrations of dyestuff and water, and concluded that the radius of the dyestuff micelle is approximately 4.9×10^{-8} cm. and that this is surrounded by a water shell 17.6×10^{-8} cm. in thickness. The effective radius of the micelle and the water shell accordingly is

[4] Liepatoff, S. von, Ueber die Viskosität und Hydration von Farbstofflösungen, *Koll. Z.*, 39 : 230–236 (1926).

22.5×10^{-8} cm. Figure 76 shows such a dyestuff particle and the adsorbed water shell in their proportionate relationship. It is evident from such a diagram that relatively large viscosity changes may be brought about by relatively small amounts of the disperse phase, inasmuch as every added increment of the disperse phase combines with a much larger amount of the dispersions medium, removing it from a mobile to a more or less fixed state.

That there is a certain packing of the molecules of the dispersions medium in the shell surrounding the micelles is evidenced by the fact that a volume change occurs in the formation of a gel. As a rule a contraction of the system takes place so that the volume of the gel is less than the volume which would be occupied by the dry disperse phase and the pure dispersions medium. Various workers have studied this problem. Chick and Martin[5] reached the conclusion that the density of protein micelles in sols is from 5 to 8 per cent greater than the density of protein in the dry state. Svedberg[6] in a more exact series of studies finds a contraction in the system of about 54 cu. mm. per gm. of gelatin dissolved in water at 35°. The greatest initial contraction occurs with the initial increments of water which are added. The contraction increases with a decreased temperature which apparently indicates an adsorption reaction. It is doubtful if there is in reality an increase in the density of the gelatin micelles, as suggested by Chick and Martin, the more probable explanation being that the water molecules are oriented on the surface of the protein micelles, and in this oriented state they occupy a smaller volume than do the water molecules which are in the bulk of the liquid.

That gels possess a structure and that there are attraction forces between the disperse phase and the dispersions medium is further evidenced by the work of Holmes, Kaufmann, and Nicholas.[7] They allowed silica gels to set in test-tubes of the same length but of varying diameters. When the tubes containing the gels were tapped, sound-producing vibrations were set up which were related not to the length of the column of the gel but rather to the diameter of the gel column. A vibration frequency of 341 per second was found for a column of gel 34 mm. in diameter, increasing to 640 vibrations per second for a column 23 mm. in diameter, and further increasing to a frequency of 1152

[5] Chick, H., and Martin, C. J., Die Dichte und das Lösungsvolumen einiger Proteine, *Koll. Z.*, 12 : 69–71 (1913).

[6] Svedberg, The, Density and Hydration in Gelatin Sols and Gels, *J. Am. Chem. Soc.*, 46 : 2673–2676 (1924).

[7] Holmes, H. N., Kaufmann, W. E., and Nicholas, H. O., The Vibration and Syneresis of Silicic Acid Gels, *J. Am. Chem. Soc.*, 41 : 1329–1336 (1919).

vibrations per second when the column was only 15 mm. in diameter. An increase in the concentration of the silica gel increased the pitch of the vibration. Undoubtedly these vibrations were due to a rigidity of gel structure. If the test-tubes were previously greased so that the gel could not adhere to the glass, no vibration was observed. When silica gels in a state of tension similar to the above " musical " gels are allowed to stand for any considerable period of time, they are usually torn apart by their own internal stresses, in some instances with such force as to shatter the wall of the tube enclosing them. In such gels the entire system reacts as a rigid solid, indicating that the disperse phase has bound all or nearly all of the dispersions medium.

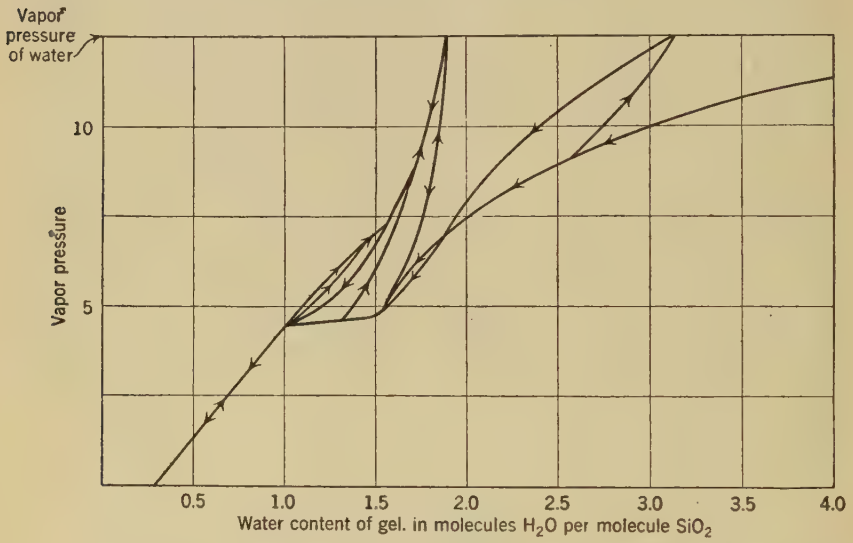


FIG. 77.—Hydration ⇆ dehydration curves of silica gel. (Data of van Bemmelen.)

Van Bemmelen[8] in his classic study of silica gels has definitely proven that the water which is present is not water of constitution, and he could secure no definite evidence of the presence of any hydrates of SiO_2. He did show that the amount of water retained by a silica gel was dependent on the past history of the gel. He found that the dehydration curve of silica gel was a continuous process but was not an equilibrium. A silica gel which has been dehydrated to a certain point will again take up water if placed in a suitable environment, but the rehydration curve will not follow the dehydration path. Figure 77 shows the familiar dehydration-hydration curves of silicic acid gels as found by van Bem-

[8] van Bemmelen, J. M., Die Absorption, Theodor Steinkopff, Dresden (1910).

melen. The arrows pointing downward indicate the dehydrating processes, the arrows pointing upward indicate the hydrating processes. It will be noted that, as a rule, gels which have been partially dehydrated have a lowered capacity for reimbibing water. This is characteristic not only of silica gels but of all, or nearly all, of the organic gels. The aging of a gel is similarly characterized by a decrease in the intensity of the forces with which the dispersions medium is held. This is another point at which the *time* factor enters into colloidal behavior and is usually one of the most striking examples of hysteresis.

Throughout van Bemmelen's work no evidence for definite hydrates of SiO_2 was found in spite of the fact that an SiO_2 surface strongly attracts molecules of water. This is probably due to the fact that the SiO_2 surface is a polar surface and water is a polar liquid.

The tenacity with which water is held upon the surface of aluminum oxide has already been referred to. An aluminum oxide gel which has been heated to 700 to 750° is one of the most efficient drying agents known and is used in organic combustions in place of calcium chloride or P_2O_5. Johnson[9] states that "1 gram of Al_2O_3 can practically completely absorb all moisture from approximately 10 liters of air saturated with water vapor at 18°." Later studies have shown that aluminum oxide which is perfectly anhydrous will not act as an efficient adsorbent for water but instead the adsorbing properties depend upon an aluminum gel from which all of the original water has not been removed.

The terms, solvation, hydration, and imbibition, are used interchangeably in referring to the process by which water is taken up by the bio-colloids.

Methods for Measuring Imbibition.—Three general methods have been used for the measurement of imbibition by lyophilic colloids, (1) the swelling of granules of the lyophilic colloid when placed in water, (2) the increase in weight of the lyophilic colloid when placed in water or in atmospheres of varying humidity, and (3) changes in viscosity of the system containing the lyophilic colloids.

The method for increase in volume has been used very largely by Martin Fischer in his studies of the hydration of fibrin and other animal proteins. Selecting test-tubes of a uniform bore, a given amount of the protein is placed in the tube and the various solutions whose effect on imbibition it is desired to study, are added. At varying intervals of time the height of the swollen column of protein is measured, and the amount of imbibition which has taken place is calculated from the height of the swollen column.

[9] Johnson, F. M. G., Alumina as a Drying Agent, *J. Am. Chem. Soc.*, 34 : 911–912 (1912).

Hofmeister was probably the first to use the increase in weight method. In such studies a sheet of gelatin or some similar lyophilic colloid is placed in the liquid to be studied and from time to time is removed and weighed. The increase in weight plotted against time will give an imbibition curve. This method has been used by Upson and Calvin[10] and by Gortner and Doherty[11] in studying the quality of wheat-flour proteins.

Wo. Ostwald[12] notes among other factors that viscosity increases with increasing solvation of lyophilic colloids. Gortner and Sharp [13,14] and Lüers and Ostwald[15] almost simultaneously applied the viscosity method to the study of wheat-flour proteins, and in a later paper Lüers and Schneider[16] carried out a comparative study in which Hofmeister's method of weighing and Fischer's method of volume change were compared with the viscosity method. They reached the conclusion that all three methods are equally suitable for measuring hydration capacity. The viscosity method permits of greater ease of manipulation and of a considerably higher degree of accuracy. Accordingly it seems the preferable method to employ whenever experimental conditions permit of its use.

Imbibition Pressure.—Many organic colloids show a great affinity for certain liquids and will take up such liquids against relatively enormous pressures. The pressure against which such a colloid will imbibe a liquid, or conversely the pressure which is required to force the dispersions medium out of a gel is known as the imbibition pressure. Imbibition pressures should not be confused with osmotic pressure, and in many instances they assume values greatly in excess of values obtainable by osmotic pressure. Thus, for example, dry seeds will, through imbibition

[10] Upson, F. W., and Calvin, J. W., On the Colloidal Swelling of Wheat Gluten, *J. Am. Chem. Soc.*, 37 : 1295–1304 (1915).

[11] Gortner, R. A., and Doherty, E. H., Hydration Capacity of Gluten from "Strong" and "Weak" Flours, *J. Agr. Res.*, 13 : 389–418 (1918).

[12] Ostwald, Wo., Ueber die Bedeutung der Viskosität für das Studium des kolloiden Zustandes, *Koll. Z.*, 12 : 213–222 (1913).

[13] Gortner, R. A., and Sharp, P. F., The Physico-Chemical Properties of Strong and Weak Flours. III. Viscosity as a Measure of Hydration Capacity and the Relation of the Hydrogen Ion Concentration to Imbibition in the Different Acids, *J. Phys. Chem.*, 27 : 481–492 (1923).

[14] Sharp, P. F., and Gortner, R. A., Viscosity as a Measure of Hydration Capacity of Wheat Flour and its Relation to Baking Strength, Technical Bulletin No. 19, Minnesota Agricultural Experiment Station, 119 pp. (1923).

[15] Lüers, H., and Ostwald, Wo., Beiträge zur Kolloidchemie des Brotes V. Die kolloide Quellung des Weizenklebers, *Koll. Z.*, 27 : 34–37 (1920).

[16] Lüers, H., and Schneider, M., Zur Messung der Solvatation (Quellung) in Kolloiden, *Koll. Z.*, 28 : 1–4 (1921).

pressure, withdraw water from a saturated solution of lithium chloride, which has an osmotic pressure of approximately 1000 atmospheres, although the salt content of the seed is sufficient to account for only a few atmospheres of osmotic pressure. Similarly, if a sheet of dried gelatin is placed in a saturated solution of sodium chloride, water will be withdrawn by imbibition forces against the osmotic pressure of the sodium chloride solution, and sodium chloride will crystallize out in the solution. The sap of plants in salt marshes or alkali flats may reach an osmotic pressure as high as 172 atmospheres[17] (14.4° depression of freezing point), but such plants are not characteristic of the more extreme

FIG. 78.—*Opuntia Sp.* growing in 3 inches of soil on the roof of an adobe house near Tucson, Arizona. Annual rainfall about 10 inches.

xerophytes, such as the cacti, where the osmotic pressure plays little or no role, and imbibition pressure becomes all-important.

Figure 78 shows a cactus (*Opuntia sp.*) growing in 3 inches of adobe soil on the roof of an Indian hut near Tucson, Arizona, in a region of approximately 10 inches of annual rainfall. The osmotic pressure of such cacti probably does not exceed 6 or 7 atmospheres as measured by the depression of the freezing point of the sap. Nevertheless the imbibition pressure of the colloids which are present in the mucilaginous juices is so great as to withdraw water from an extremely dry soil and to resist the desiccating effects of the desert winds throughout the long periods devoid of rainfall.

[17] Harris, J. A., Gortner, R. A., Hoffman, W. F., and Valentine, A. T., Maximum Values of Osmotic Concentration in Plant Tissue Fluids, *Proc. Soc. Exp. Biol. Med.*, 18 : 106–109 (1921).

Newton[18] and his co-workers, in July, 1925, removed some stems from the Alberta *Opuntia*, sealed the cut surfaces with grafting wax, and then placed the segments in a desiccator over concentrated sulfuric acid. At the end of six months in an atmosphere of almost zero humidity, the stems had lost less than 10 per cent of the water which they originally contained. At the end of 94 days of desiccation, one of the stems was removed from over the sulfuric acid and placed in another desiccator over a free water surface. Figure 79 is a photograph of this stem taken 54 days after it had been placed in the atmosphere of higher humidity. It will be noted that two new shoots were already well developed. A change in the relative humidity of the air was sufficient to produce a growth response. It had not rained on the cactus but it had apparently rained in the immediate vicinity as indicated by the increased humidity, and the plant was ready to respond at once to favorable conditions for growth. The ability of the *Opuntia* to resume growth almost as soon as favorable conditions appear is abundant evidence that the apparently drastic desiccation over sulfuric acid was of no real significance insofar as the vital activities of the cactus were concerned but that the imbibitional forces with which the colloids of the stem retained and imbibed water constitute a vital factor in the ability of the plant to grow under extremely adverse conditions.

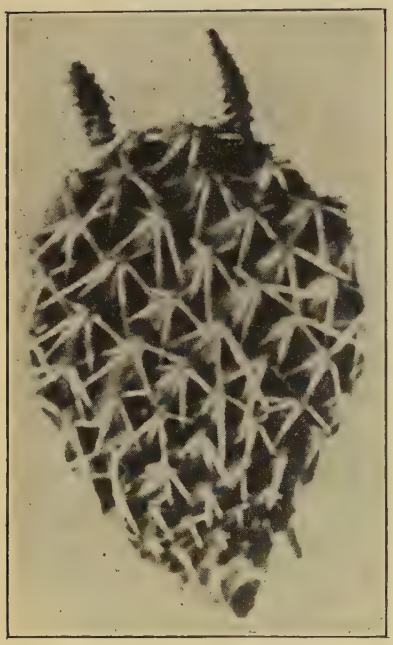


FIG. 79.—*Opuntia Sp.* removed from desiccator after ninety-four days over concentrated H_2SO_4 and placed in another desiccator over pure water. Growth was observed within two weeks of transference. Photograph taken fifty-four days after transference. (Photograph, courtesy of Dr. Robert Newton.)

Similar examples of the force with which water is taken up or retained by organic colloids are numerous. Starch will still swell against a pressure of 2500 atmospheres if heated in the presence of water. Reinke,[19]

[18] Unpublished data.

[19] Reinke, J., Untersuchungen ueber die Quellung einiger vegetabilischer Substanzen, *Hanstein's botanische Abhandl.*, 4 : 1–137, 4 plates, (1879).

in 1879, measured the swelling pressure of dried disks of the sea algae, *Laminaria*, against water. He placed disks of the dried *Laminaria* in a hollow metal cylinder, and above the disks fitted a metallic piston carry-

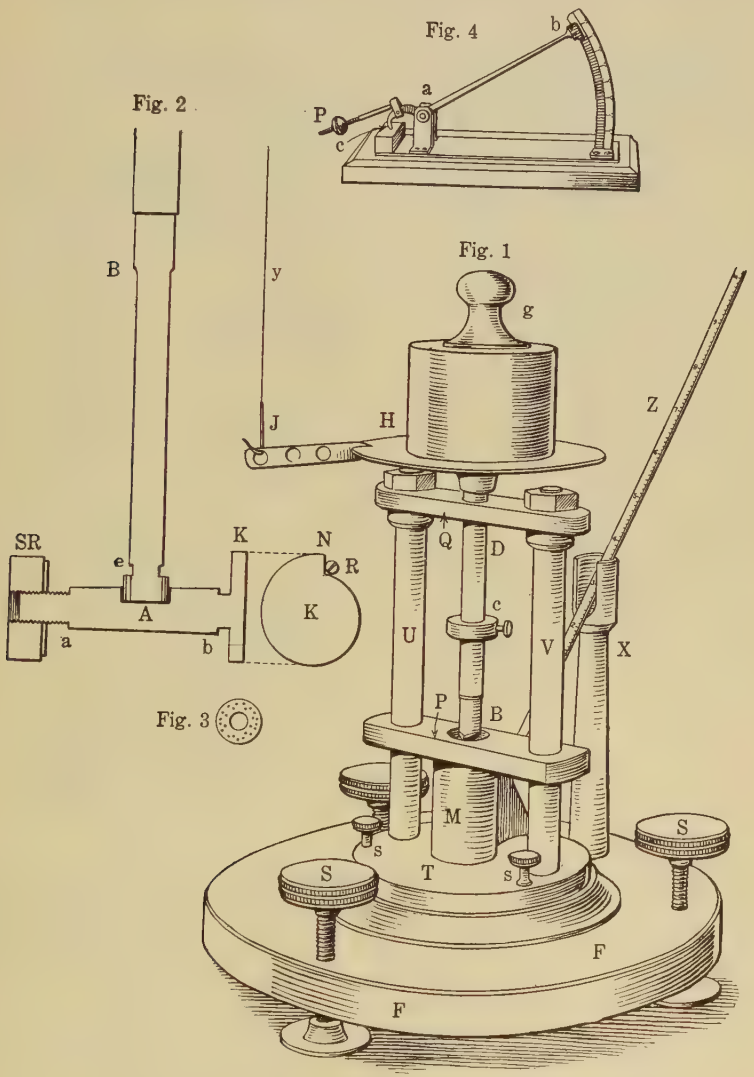


FIG. 80.—Reinke's original apparatus for measuring imbibition pressure.

ing a platform upon which weights could be placed. The disks of dried algae were then allowed to come in contact with water, and the amount of swelling against known weights was measured by the movement of the

piston. When no further swelling took place, a part of the weights could be removed, and swelling again set in, reaching a new equilibrium determined by the weight on the piston. Figure 80 shows the

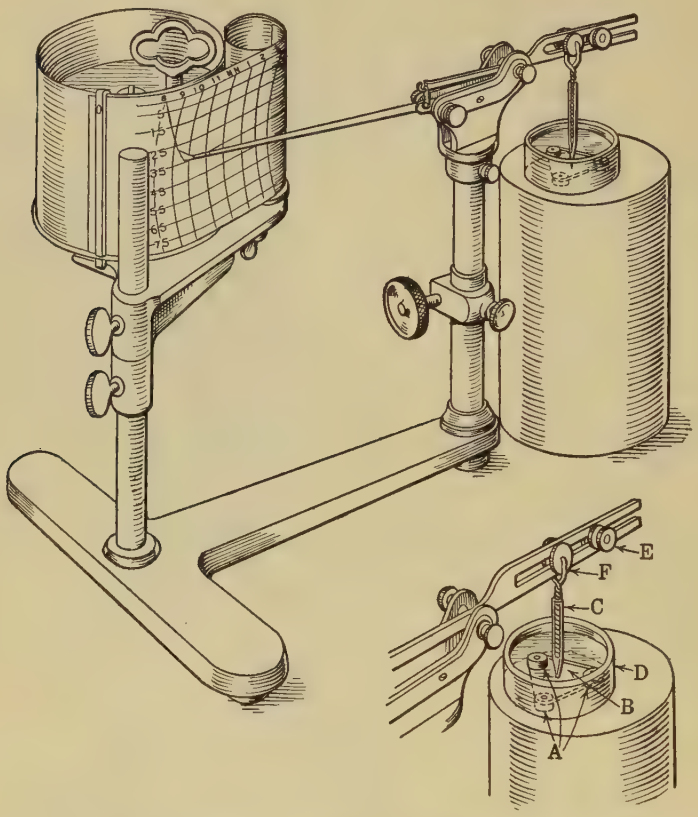


FIG. 81.—MacDougal's auxograph arranged for recording changes in thickness, due to swelling, of trio of sections of plant tissues and of biocolloids. The vertical arm, which is set in position on horizontal arm to give an amplification of 20, rests on a triangle of glass laid on top of the sections. The dish containing the sections rests on an iron cylinder to secure stability and a weight is placed on the T base of the instrument. The record sheet is ruled to millimeters (not shown) with heavier horizontal lines 1 cm. apart. The heavy curved lines represent hour intervals. The space is ruled to 15-minute intervals (not shown). Height of clock and lever supports adjustable.

original apparatus used by Reinke in these pioneer studies of imbibition pressure.

That imbibition pressures are not limited to hydrophilic colloids

is evidenced by the work of Posnjak,[20] who studied the swelling pressures of rubber in organic liquids and gelatin in water. He found the same general phenomena in both types of systems.

MacDougal[21] has greatly refined the methods of measuring the swelling of bio-colloids by constructing an auxograph, the essential parts of which consist of a compound lever, one end of which rests upon the swelling material, and to the other end a pen is attached which traces a continuous imbibition curve upon paper fastened to a rotating drum. The general arrangement of the apparatus is shown in Fig. 81. By the use of this instrument MacDougal and his co-workers have studied various problems involving the swelling of plant colloids, and in his monograph MacDougal attempts to correlate the observations with the growth processes.

Heat of Imbibition.—We have already discussed in Chapter VI the question of heat of adsorption. Imbibition can be regarded as the adsorption of the dispersions medium, and accordingly the heat of imbibition is only a special example of heat of adsorption. What has been said under heat of adsorption will accordingly apply equally well to heat of imbibition. We have noted that there is a contraction of the system when a gel imbibes the dispersions medium, and we have likewise noted that there is a positive imbibition pressure. The volume contraction is directly related to the imbibition pressure and if there is a positive imbibition pressure, there must be an evolution of heat under such pressure. Accordingly one can construct an imbibition curve by following either the volume changes which take place during imbibition or by following an imbibition pressure curve or by following the temperature changes which occur during the imbibition process.

Imbibition should not be confused with solution. The imbibition process is in general characterized by an evolution of heat. On the contrary, the solution process is in general characterized by an absorption of heat. Thus, in the case of gelatin the initial swelling process liberates heat, whereas the final dispersing process absorbs heat. From the heat which is liberated it is possible to reconstruct the imbibition curve. The curves so constructed are typical adsorption curves, being parabolas when the numerical values are plotted and straight lines when the ordinates and abscissas are logarithmic values.

Rate of Imbibition and Maximum Imbibition Capacity.—The determination of the maximum imbibition capacity is in some instances

[20] Posnjak, E., Ueber den Quellungsdruck, *Kolloidchem. Beihefte*, 3 : 417–456 (1912).

[21] MacDougal, D. T., Hydration and Growth, Carnegie Institution of Washington Publication No. 297, Washington (1920).

of the utmost importance in detecting differences in the degree of colloidality of lyophilic colloids. It has been used by Gortner and Doherty [22] and by Sharp and Gortner [23] in studies dealing with the quality of the gluten proteins in wheat flour.

Those flours which are superior for the manufacture of yeast-leavened bread are known to the trade as " strong " flours. The National Association of British and Irish Millers have adopted the definition of a strong wheat as " one which yields flour capable of making large, well-piled loaves. The latter qualification thus excludes those wheats producing large loaves which do not rise satisfactorily."

Gortner and Doherty studied a series of glutens from wheat flours differing in baking strength, by following the increase or decrease of water imbibition occurring when weighed disks of gluten were immersed in acid solutions of various concentrations. They found that the glutens from strong flours showed a much more rapid rate of imbibition than did glutens from weak flours, *i.e.*, there was a more rapid increase in weight in a unit period of time. An even more striking observation, however, was the fact that the glutens from strong flours had a much greater maximum imbibition capacity than did the glutens from weak flours and that the strong glutens could take up a relatively large amount of water and still retain their coherence, whereas the weak glutens were rapidly peptized and changed from a gel to a sol at a much lower degree of hydration. After sixty minutes' immersion in certain of the solutions, the gluten disks from the weak flours had lost their coherence, although the disks had imbibed less than 100 per cent of their original weight. The strong glutens on the other hand still remained coherent at the end of a two-hour period, in which time they had imbibed nearly 300 per cent of their original weight. Sharp and Gortner reached similar conclusions, using not only the method of increasing weight for measuring imbibition, but in later studies using viscosity methods. The studies may be summed up in the statement of Gortner and Doherty that, " the difference between a strong and a weak gluten is apparently that between a nearly perfect colloidal gel with highly pronounced physicochemical properties such as pertain to emulsoids and that of a colloidal gel in which these properties are much less marked."

Probably many other systems could be studied to equal advantage by observing not only the rate of imbibition but the maximum degree of

[22] Gortner, R. A., and Doherty, E. H., Hydration Capacity of Gluten from "Strong" and "Weak" Flours, *J. Agr. Res.*, 13 : 389–418 (1918).

[23] Sharp, P. F., and Gortner, R. A., Physico-Chemical Studies of Strong and Weak Flours. II. The Imbibitional Properties of the Glutens from Strong and Weak Flours, *J. Phys. Chem.*, 26 : 101–136 (1922).

solvation which is possible before imbibition reaches a point where the gel passes into a sol.

The Effect of Hydrogen Ion Concentration on Imbibition.—Protein systems show a remarkable effect of hydrogen ion concentration on rate of imbibition and maximum imbibition capacity. The preliminary work in this field was carried out by Martin Fischer [24] in an attempt to apply colloid studies to pathological problems. Fischer's early work was carried out before the importance of hydrogen ion concentration in biological systems was stressed and before hydrogen ion concentration measurements were a part of the technic of the biochemical laboratory. Accordingly most of Fischer's imbibition curves were drawn with normality of acid concentration as one of the variables rather than hydrogen ion concentration. The curves which Fischer presents are very similar to those which have already been presented in Fig. 34, and he noted that the various acids produced very dissimilar curves. The lack of similarity in the curves was due to the fact that the various acids with which he worked had very different hydrogen ion concentrations at equivalent normalities, and had his curves been drawn with respect to a single variable, *i.e.*, the hydrogen ion concentration, they would all have possessed a similar shape as shown in Fig. 35.

Since these early studies of Fischer, many other workers have studied the swelling of proteins in various acid solutions at different concentrations. The work of Gortner and Doherty and of Sharp and Gortner on wheat flour proteins has already been referred to, and their data may be taken as typical examples of the results to be obtained in protein systems. Similarly, the work of Loeb [25] has stressed the importance of hydrogen ion concentration as one of the major factors in the swelling of proteins in acid or alkaline solutions.

It would take us too far afield to list all or even the more important papers having to do with the effect of hydrogen ion concentration on imbibition in protein systems. There seems, however, to be a unanimity of opinion that a minimal degree of swelling occurs in solutions where the protein is in an isoelectric condition. At hydrogen ion concentrations on either side of the isoelectric point there is a rapid increase in imbibition which reaches a maximum on the acid side at approximately *p*H 2.5–3.0 and on the alkaline side at approximately *p*H 10.5. In solutions more acid than *p*H 2.5 or more alkaline than *p*H 10.5, there is

[24] Fischer, Martin H., Oedema and Nephritis, Third Edition, John Wiley and Sons, Inc., New York (1921).

[25] Loeb, J., Proteins and the Theory of Colloidal Behavior, McGraw-Hill Book Company, Inc., New York (1922). (Cf. also numerous papers in the Journal of General Physiology, Vols. 1–7, inclusive.)

usually a sharp decrease in imbibition capacity so that an imbibition curve of protein in acid or alkali more or less resembles the diagrammatic peptization curve of silver bromide as noted in Fig. 6. The increase in imbibition up to *p*H 2.5 and 10.5 is probably due to an increase in the electrical forces making up the Helmholtz double layer, whereas the decrease in higher concentrations of acid or alkali may very well be dehydrating effects due to the very appreciable concentration of electrolytes in the dispersions medium.

MacDougal[26] has shown that in contrast to protein systems the

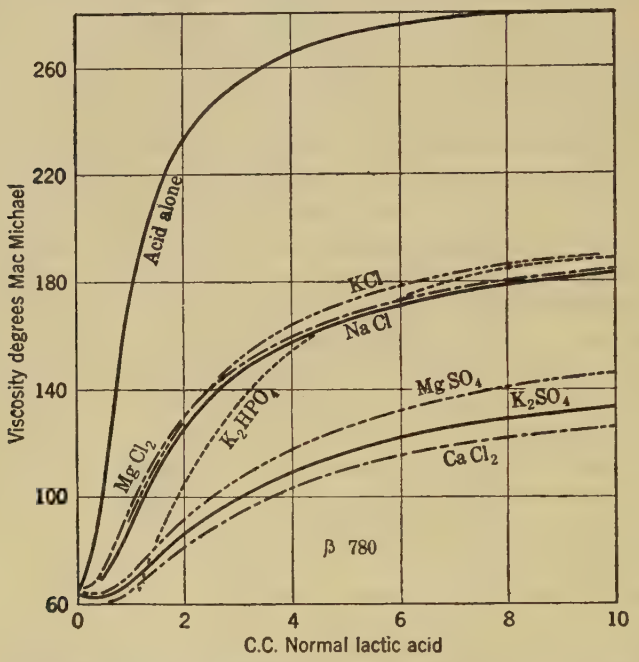


FIG. 82.—Imbibition curves as measured by the viscosity of a 20 per cent flour-in-water suspension made 0.01 N with respect to various salts and with the subsequent addition of various amounts of lactic acid. (Data of Gortner and Sharp.)

carbohydrate gels, such as agar, cactus mucilage, etc., have a maximum imbibition in solutions which are essentially neutral and that at hydrogen ion concentrations differing appreciably from the neutral point there is a decreased imbibition capacity. He finds that the imbibition curves of plant tissues can be duplicated by mixtures of carbohydrate and protein, and suggests that the curves characteristic of plant tissues are due

[26] MacDougal, D. T., Hydration and Growth, Carnegie Institution of Washington Publication No. 297, Washington (1920).

to a mixture of approximately 9 parts of a polysaccharide, such as agar, and 1 part of a protein. The surprising thing in regard to MacDougal's observations is that very dilute solutions of amino acids favor imbibition of his "bio-colloids" to a rather remarkable degree.

The Effect of Salts on Imbibition.—Neutral solutions of electrolytes influence imbibition in a manner very similar to their effect upon lyophobic sols. Fig. 82, taken from the data of Gortner and Sharp,[27] shows the decrease in viscosity of an acidulated wheat flour-water suspension to which various salts in 0.01 N concentration have been added. It will be noted that there is here evidence of a distinct lyotropic series. As already noted, the original lyotropic series of Hofmeister was based upon observations of the swelling of proteins in various solutions. Bayliss[28] has presented an excellent discussion of the role that colloid chemistry plays in physiology and (pp. 137–143) devotes a considerable part of the discussion to the relation of electrolytes to the biochemical colloids.

Syneresis.—Syneresis is, in some respects at least, the reverse of imbibition, a part of the liquid which has been imbibed by a gel being squeezed out of the gel either by a contraction of the gel structure or under the influence of external conditions which decrease the imbibition capacity of the gel. The squeezing out of a serum from a blood clot, the "bleeding" of an agar slant, the liquid which collects around the mold of gelatin in an ice box, the "breaking" of custards, the separation of whey when milk sours, or in the cheese manufacturing process, the "sweating" of bread, "leaky" butter, the separation of liquid from lean meat when heated, and the serum that exudes from a wound or blister are all typical examples of the phenomenon of syneresis.

Wo. Ostwald states that syneresis is one of the most characteristic properties of the gels. The liquid which exudes from the gel is not pure water but rather is a dilute sol, the composition of which is determined by the composition of the original gel. Healy[29] has studied the composition of the liquid of syneresis as related to the original composition of the nutrient agar and finds that the liquid of syneresis is an excellent medium for bacterial growth.

Inasmuch as protoplasm is a true colloid system, it must "secrete" when it contracts. Undoubtedly the liquid in the contractile vacuole is

[27] Gortner, R. A., and Sharp, P. F., The Physico-Chemical Properties of Strong and Weak Flours. IV. The Influence of the Ash of Flours upon the Viscosity of Flour-in-Water Suspensions, *J. Phys. Chem.*, 27 : 567–576 (1923).

[28] Bayliss, W. M., Colloid Chemistry in Physiology, Second Report on Colloid Chemistry, pp. 117–154, Brit. Assoc. for the Adv. Sci., London (1918).

[29] Healy, D. J., The Exudate from Nutrient Agar Slants, The So-Called Water of Condensation, *J. Bact.*, 12 : 179–180 (1926).

liquid of syneresis. One theory of gland secretion has been built up on imbibition followed by syneresis, and affords the most satisfactory explanation for the phenomena which are observed. Similarly, a theory of muscle contraction involves imbibition followed by syneresis. As the nerve impulse reaches the muscle fiber, carbohydrates are partially burned, giving rise to the intermediate lactic acid. The increased hydrogen ion concentration favors imbibition of the muscle fiber (sarcostyle) which is bathed by the fluid, sarcoplasm. Water is accordingly imbibed from the sarcoplasm and because of the anatomical structure of the sarcostyle, the increase in volume causing thickening and shortening of the fiber, resulting in contraction. As the nerve impulse passes, the lactic acid is either further burned to carbon dioxide and water or is recombined into glucose or glycogen. The muscle fiber now holds an excess of water above its maximum imbibition capacity at the prevailing hydrogen ion concentration, and accordingly liquid is again lost by syneresis to the sarcoplasm, causing a relaxation of the muscle. Hofmeister points out that the time element is not inconsistent with the necessary sequence of imbibition followed by syneresis. A bee's wing may vibrate as rapidly as 200 times per second. Hofmeister has calculated that protein strands as fine as the fibriles in the muscle of a bee's wing may easily imbibe sufficient liquid and undergo syneresis within the time limits necessary to account for the observed rate of contraction and relaxation. A contracted muscle invariably has a higher water content than does a relaxed muscle, and while imbibition and syneresis are probably not the only factors which are involved, they certainly are contributing forces.

Rigor mortis appears to be due to the imbibition of water by the protein structure, following the accumulation of lactic acid in the tissues, and can be prevented if the formation of appreciable quantities of lactic acid is inhibited. The death of a multicellular organism is in reality only the loss of coordination by the various cells and organs of the body. Many of the cells remain alive for longer or shorter periods after the organism is dead, such cells being eventually killed by asphyxiation or by the accumulation of waste products due to a failure of the circulatory system. So long as a cell remains alive, respiration persists. Lactic acid and carbon dioxide, being acid products of respiration, cause an increased hydrogen ion concentration which favors the imbibition of protein tissues. The water is taken up, and the muscle sets to a rigid gel. That rigor mortis is related to cell oxidation is evidenced by the fact that it is hastened if the body is placed in an atmosphere of oxygen and is retarded or inhibited if it is placed in an atmosphere of hydrogen or nitrogen. Similarly, an organism will very rapidly undergo rigor

mortis if placed in an acid atmosphere of carbon dioxide or if injected with dilute acid solutions, whereas rigor mortis will be inhibited if the organism is placed in an atmosphere of dilute ammonia or injected with alkaline solutions.

The Aging of Gels.—The *time* factor affects all colloid systems but is particularly noticeable in gel studies. The micelles may aggregate into larger units, or crystal growth may be a factor, and the larger particles may grow at the expense of the smaller ones, due to an unequal distribution of surface energy forces. With the bio-colloids there is a decrease in imbibition capacity with age. Probably the changes involved in senescence are in a large measure changes characteristic of aged colloid systems. The older leaves on a tree are characteristically more lignified and contain a lower content of colloids in the sap which can be expressed than do the leaves which are just unfolding. Old tissues, both of plants and of animals, are in general less highly hydrated than are the younger tissues. The walls of the blood vessels of older animals contain a higher proportion of dry matter, a higher proportion of inorganic constituents, notably calcium, and a much lower proportion of water, than do walls of the blood vessels of young animals.

The body tissues can be looked upon as examples of colloid gels, the behavior of which may be expected to be intimately related to the gel structure and the water content. In the hardening of the arteries (*arterio sclerosis*) of old age, we have a striking example of the loss of flexibility of the arterial wall which is associated with a higher content of dry matter and a reduced imbibition capacity. Thoenes[30] has likewise shown that the muscle tissues of dogs and guinea pigs have a progressively lowered imbibitional capacity as the animal becomes older. If the problems of rejuvenescence are ever solved, they will be solved very largely through colloid chemical studies designed to bring about an increased, or to maintain a high, imbibition capacity of the tissue colloids.

The Role of Water in the Living Organism.—Inasmuch as living organisms are very largely composed of water, a study of the gels which comprise the living organism is extremely important to the biologist.

Table XXX gives the approximate elementary composition of the human body, as well as the relative proportion of water, protein, fat, salts, and other organic compounds. It will be noted that the greater percentage of the human body is composed of water. This is probably true of every living organism. In some instances, as in the case of the jelly fish, only an insignificant fraction of the organism is composed of organic material, as little as 1 per cent of the jelly fish being organic

[30] Thoenes, F., Untersuchungen zur Frage der Wasserbindung in Kolloiden und tierischen Geweben, *Biochem. Z.*, 157 : 174–186 (1925).

TABLE XXX

Composition of the Human Body

Elementary Composition		Group Composition	
	Per Cent		
Oxygen	66.0		
Carbon	17.5		
Hydrogen	10.2		
Nitrogen	2.4		
Calcium	1.6		Per Cent
Phosphorus	0.9	Water	65.0
Potassium	0.4	Protein	15.0
Sodium	0.3	Fat	14.0
Chlorine	0.3	Salts	5.0
Sulfur	0.2	Other organic compounds	1.0
Magnesium	0.05		
Iron	0.004		
Iodine, Fluorine, Silicon, Manganese, Arsenic, etc	Present		

matter. Figure 83 illustrates what a small proportion of dry matter enters into the composition of a jelly fish. In this figure the " bell " of a jelly fish has been dried down upon the page of a magazine and the print can be rather readily seen through the dry material of what was formerly a living organism. It has been said that, " the sea ebbs and flows through a jelly fish," which is essentially saying that the water of a jelly-fish structure plays no particular function. Undoubtedly this is incorrect. The many striking and unusual results which one obtains in dealing with dilute gels of the lyophilic colloids cannot be duplicated when one works with the dry materials, and the jelly fish must be thought of as a biological system in which water plays a role and probably a more important role than any other single constituent.

In most organisms a very considerable part of the growth process is nothing more nor less than an imbibition of the bio-colloids. For example, a frog's egg, weighing on the dry basis only a few milligrams, can, after fertilization, be placed in a dish of filtered, sterile water and allowed to undergo the process of development. Such an egg will undergo cell division, giving rise at the end of several weeks to a living tadpole which may be as much as 2 centimeters in length and weighing several grams. Such a tadpole has never partaken of food other than utilizing the nutrients already present in the original egg and will be found on analysis

to contain less dry matter than the original egg, due to the fact that a certain amount of the organic materials present have been utilized as a source of energy and eliminated as carbon dioxide and water. The growth during these several weeks has all been due to the intake of liquid water which has become "living water," so the tadpole is actually more than 99 per cent water. It would be ridiculous to speak of this organism as being composed of only 1 per cent of vital materials. The water is as much a part of the tadpole as are the fats, proteins, etc., which serve to form the gel structure, and the biochemical and biophysical reactions which take place within the cells and tissues of the tadpole are determined probably more by the water which is present than by any or all of the other constituents.

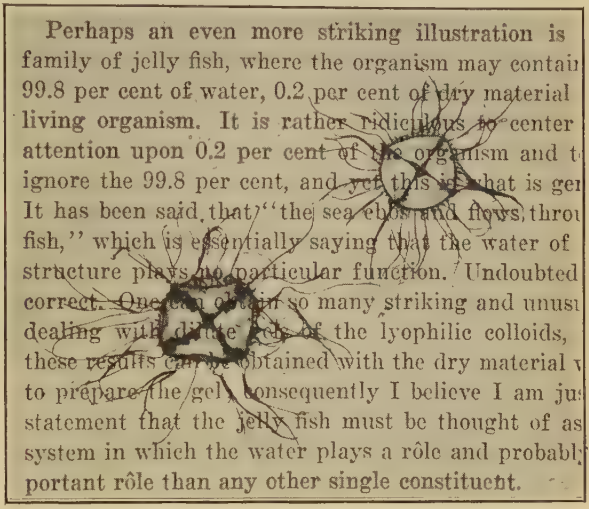

Perhaps an even more striking illustration is
family of jelly fish, where the organism may contai
99.8 per cent of water, 0.2 per cent of dry material
living organism. It is rather ridiculous to center
attention upon 0.2 per cent of the organism and t
ignore the 99.8 per cent, and yet this is what is ge
It has been said that "the sea ebbs and flows throu
fish," which is essentially saying that the water of
structure plays no particular function. Undoubted
correct. One can obtain so many striking and unusu
dealing with little cells of the lyophilic colloids,
these results can be obtained with the dry material
to prepare the gel. Consequently I believe I am ju
statement that the jelly fish must be thought of as
system in which the water plays a rôle and probabl
portant rôle than any other single constituent.

FIG. 83.—Showing adult medusae (*Gonionemus Sp.*) dried down upon a magazine page. It will be noted that the amount of dry material in the umbrella is so slight that the print can easily be read through the dried organism. The dark cross bars underneath the umbrella are the radial canals to which are attached the reproductive organs, which in these specimens are filled with ripe ova.

The constituents that go to make up a living organism may be classified into five great groups, (1) proteins, (2) carbohydrates, (3) fats and lipides, (4) the inorganic salts, and (5) water. There are, to be sure, a number of compounds which do not fall definitely within one or another of these groups, but the actual amounts of such compounds are extremely small.

Of these various groups, the proteins belong to the class of lyophilic colloids. They possess the power of becoming strongly hydrated, and the large amount of water which is present in living organisms is probably mainly held through this affinity of the proteins for water.

Insofar as cell protoplasm is concerned, carbohydrates can be largely looked upon as a source of energy, the carbohydrate content of true pro-

toplasm being relatively low. In the plant kingdom, however, the structural elements and intracellular constituents are largely of carbohydrate nature, polysaccharides in most instances. These polysaccharides are in many cases highly hydrophilic and combine with relatively large amounts of water.

The fats and lipides of living organisms exist in the form of emulsions. In the study of such emulsions, attention has been chiefly directed toward the fats, and relatively little attention has been given to the aqueous phase. Adipose tissue usually contains as much, if not more, water than it does fat, and in some instances it is possible to break down adipose tissue by the use of large doses of drastic diuretics more readily than to reduce obesity in any other way. Certain of the phosphatides, such as lecithin, are in themselves extremely hydrophilic and exist in the colloidal state and react as typical lyophilic colloids.

The salts and other true solutes present in the biological organism may be, to a very considerable extent, molecularly dispersed in the water and, on the other hand, may exist in a more or less fixed condition by being adsorbed upon the surface of the bio-colloids. If they are adsorbed, they must be regarded as a part of the colloid system, the reaction of the surface upon which they are adsorbed being modified accordingly.

As already indicated, the water in a biological organism may exist in part as liquid water containing the truly dissolved solutes and providing the dispersions medium for the gels and sols making up the organism. A large part of the water, however, is " bound " by the colloidal micelles, and in this bound condition may exhibit entirely different properties from water in bulk.[31] Within the last few years more and more attention has been given to methods whereby this bound fraction of the water can be differentiated from the total moisture content. Every organ and cell of a biological organism has a definite fluid content and a turgidity which is regulated by the bio-colloids present in that organ or cell. In

[31] Balcar, Sansum, and Woodyatt (Balcar, J. O., Sansum, W. D., and Woodyatt, R. T., Fever and the Water Reserve of the Body, *Arch. Internal Med.*, 24 : 116–128 (1919)), proposed, under the subtitle, "A Physicochemical Theory of Fever," the theory that a bound $\rightleftharpoons$ free water equilibrium existed in the normal body tissues and that any disturbance of this equilibrium would result in bringing about certain pathological conditions.

Their theory in its essential features is very similar to the theory which has been developed by the author and which will be presented in detail in the following pages. It was only after this chapter had been completed that the paper of Balcar, Sansum, and Woodyatt came to the attention of the author. This note is accordingly inserted in order to credit these workers with what appears to be the first suggestion of the importance of a bound $\rightleftharpoons$ free water equilibrium as a factor in vital phenomena.

some instances a considerable variation in water content and turgidity may take place and life still be possible. In other instances rather minor changes will cause the death of a cell.

In the fall of 1921, Robert Newton, at the Minnesota Agricultural Experiment Station, began a series of investigations on the chemical and chemico-physical properties of winter wheat plants in order to see whether or not he could differentiate any property which would account for winter hardiness. Material for analysis was collected on October 9, and November 9, 1921. A study of the leaves and plant saps expressed from the leaves of these collections did not disclose any appreciable differences between hardy and non-hardy strains. On December 9, following the onset of winter temperatures, a third collection was made. In this study, use had been made of the technic developed by Gortner and Harris[32] for the expression of the sap from plant tissues in which permeability of the cell walls was brought about by a preliminary freezing with ice and salt, and in the collections of October and November no difficulty had been experienced in expressing approximately 60 cc. of sap from 100 gms. of the frozen wheat leaves. When, however, the collection of December 9 was tested, it was found that practically no sap could be expressed from the leaves of the hardier varieties even when the pressure employed exceeded 400 atmospheres. Thinking that perhaps permeability had not been destroyed by the preliminary freezing with an ice and salt bath, a new freezing bath was made up, using calcium chloride and snow, and the leaves were again subjected for a period of seven hours to a temperature of approximately -54° C. Even after this drastic treatment, it was found impossible to express more than 2 or 3 cc. of sap from the 100 gms. of leaves, although a moisture determination indicated that the water content of these leaves was nearly as great as the water content of the leaves of the previous collections. These observations gave a clue as to the nature of winter hardiness in wheat, and indicated that there was an elaboration of hydrophilic colloids in the plant tissues which had such a high imbibition capacity that all or nearly all of the water in the wheat leaves was in a bound condition, and in such a condition the water was either (a) no longer able to freeze, or (b) if it did freeze, did not form sufficiently large ice crystals to disrupt the protoplasm.

Newton found that the wheats that had been grown under uniform temperature conditions in the greenhouse could not be differentiated in regard to their winter hardiness by any appreciable differences in their

[32] Gortner, R. A., and Harris, J. A., Notes on the Technique of the Determination of the Depression of the Freezing Point of Vegetable Saps, *Plant World*, 17 : 49–53 (1914).

ability to hold the water against pressure, but that if they were subjected to a preliminary " hardening off " period, the amount of water which could be expressed from 100 gms. of leaves at 400 atmospheres pressure could be definitely correlated with the winter hardiness of the variety. Figure 84 shows pressure dehydration curves which Newton[33] obtained. These curves parallel field plot tests which have shown quantitatively that Minhardi is the most winter hardy of the varieties

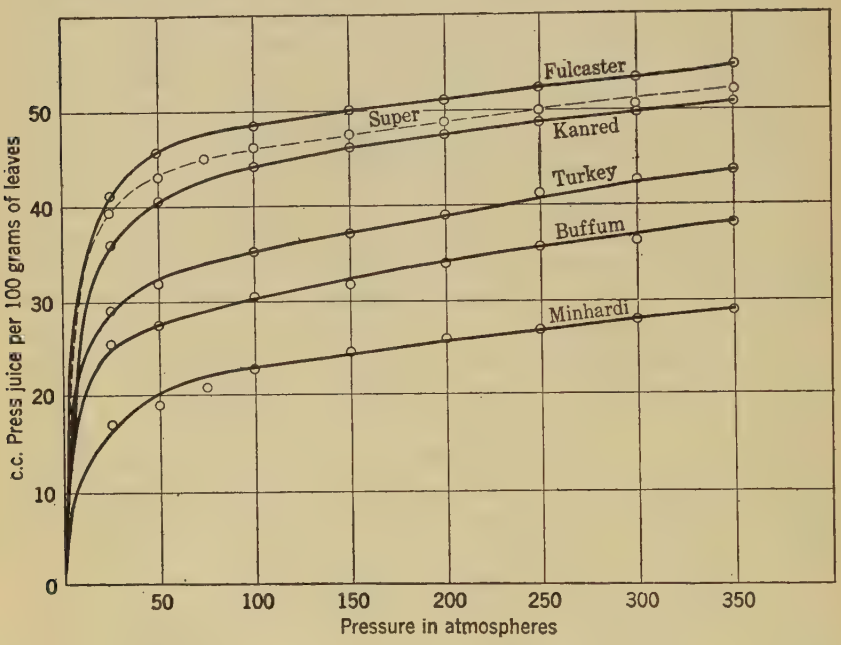


FIG. 84.—Pressure dehydration curves of winter-hardened wheat leaves. Collection made from Minnesota Agricultural Experiment Station field plots, January 21, 1922. (Data of Newton.)

tested and that Fulcaster is the least hardy. Further studies by Newton,[34, 35] and by Martin[36] have confirmed and extended the

[33] Newton, R., A Comparative Study of Winter Wheat Varieties with Especial Reference to Winter Killing, *J. Agr. Sci.*, 12 : 1–19 (1922).

[34] Newton, R., Colloidal Properties of Winter Wheat Plants in Relation to Frost Resistance, *J. Agr. Sci.*, 14 : 178–191 (1924).

[35] Newton, R., The Nature and Practical Measurement of Frost Resistance in Winter Wheat, University of Alberta, College of Agriculture, Research Bull. No. 1, 53 pp. (1923).

[36] Martin, J. H., Comparative Studies of Winter Hardiness in Wheat, *J. Agr. Res.*, 35 : 493–535 (1927).

observation that winter hardiness in wheat is intimately related to the ability of wheat varieties to bind water.

Newton and Gortner[37] attempted to devise a quantitative method whereby bound water could be differentiated from the total water present in a colloid system. Acting on the hypothesis that water which was associated with the hydrophilic colloids might not act as a solvent for sucrose, they proposed a method for the differentiation of the two forms of water.

A quantity of plant sap was weighed out which contained exactly 10 grams of water, the moisture content having been obtained by the refractometer method of Gortner and Hoffman.[38] The freezing point depression (Δ) of the plant sap had previously been taken, using the usual physico-chemical technic. To the sample of plant sap containing exactly 10 grams of water, there was then added 0.01 mole of sucrose which, if all of the water was free to dissolve sucrose, should form a molar solution of sucrose with the water which was present. The freezing point depression (Δ_a) of the sap-sucrose solution was again taken, and the difference in freezing points, due to the added sucrose, was thus determined. The molecular constant for the depression of the freezing point (K_m) was taken as 2.085° C. instead of the usual 1.86° C., inasmuch as sucrose forms a hexahydrate in solution (cf. Scatchard[39]). If less than 10 grams of water is free to dissolve sucrose, there will be a depression of the freezing point, due to the addition of the sucrose, which will be in excess of that to be expected on theoretical grounds. Accordingly if ($\Delta_a - \Delta$) is greater than 2.085° C., direct proof is obtained that all of the 10 grams of water was not free to dissolve sucrose, and the value of the excess depression is directly related to the amount of bound water. Thus, for example, 1 mole of sucrose dissolved in 1000 grams of water combines with 6 moles of water, and we have theoretically 1 mole of sucrose hexahydrate dissolved in $1000 - (18 \times 6)$, or 892 grams of water, which gives a depression of 2.085° C. (K_m).

In one of the samples of wheat sap which was worked with, it was found that the depression of the freezing point, due to the addition of 1 mole of sucrose was 2.339°. Accordingly we have $\frac{1.86 \times 1000}{2.339} = 795$ grams of water in which the sucrose hexahydrate was dissolved, and

[37] Newton, R., and Gortner, R. A., A Method for Estimating the Hydrophilic Colloid Content of Expressed Plant Tissue Fluids, *Bot. Gaz.*, 74 : 442–446 (1922).

[38] Gortner, R. A., and Hoffman, W. F., Determination of Moisture Content of Expressed Plant Tissue Fluids, *Bot. Gaz.*, 74 : 308–313 (1922).

[39] Scatchard, G., The Hydration of Sucrose in Water Solution as Calculated from Vapor-Pressure Measurements, *J. Am. Chem. Soc.*, 43 : 2406–2418 (1921).

TABLE XXXI

The Relation between Bound and Free Water of Certain Plant Saps and Lyophilic Colloid Sols

Materials Used: Leaves of	Total Solids, Per Cent	Viscosity (Water = 204), Seconds	$\triangle$ ° C.	$\triangle_a$ ° C.	$\triangle_a - \triangle$ ° C.	$\triangle_a - (\triangle + K_m)$ ° C.	Bound Water, Per Cent
Triticum vulgare var. Turkey	13.5	360	1.273	3.612	2.339	0.254	9.7
Bryophyllum calycinum	5.9	235	0.474	2.555	2.081	−0.004	0.0
Cereus sp.	4.9	637	0.505	2.803	2.298	0.213	8.3
Triticum vulgare var. Buffum	17.8	419	1.719	4.158	2.439	0.354	13.0
Triticum vulgare var. Minhardi	8.5	285	1.147	3.824	2.137	0.052	2.2
Triticum vulgare var. Super	7.1	267	1.000	3.106	2.106	0.021	0.9
Triticum vulgare var. Super	9.7	292	1.085	3.279	2.194	0.109	4.4
Sols of gum acacia							
1 per cent	1.0	309	0.005	2.147	2.142	0.057	2.37
3 per cent	3.0	487	0.013	2.186	2.171	0.086	3.53
5 per cent	5.0	684	0.025	2.221	2.196	0.111	4.50
7 per cent	7.0	932	0.034	2.254	2.220	0.135	5.42
10 per cent	10.0	1438	0.048	2.294	2.246	0.161	6.39

therefore the water bound per liter in the plant sap was 892 − 795 = 97 grams, or 9.7 per cent of the water present in the sap. The percentage of bound water is then given by the formula,

$$\frac{\text{Excess } \Delta}{\text{Observed } \Delta - \text{Sap } \Delta} \times 89.2 \tag{80}$$

or the grams of bound water equal

$$\frac{\Delta_a - (\Delta + K_m)}{\Delta_a - \Delta} \times 892 \tag{81}$$

Table XXXI shows certain of the data of Newton and Gortner. The arithmetical values for bound water in relation to concentration in sols of gum acacia when plotted form a parabola, and when the logarithms of the values are plotted a straight line results, indicating that we are dealing with an adsorption phenomenon. The curves for these values have already been given in Fig. 61.

That such a technic indicates differences in winter hardiness in wheat varieties is shown by the data of Newton as recorded in Table XXXII. This table also indicates that the elaboration of the hydrophilic colloids, or the binding of water, does not take place under greenhouse conditions but is the resultant of subjecting the plants to a period of progressively decreasing temperatures.

TABLE XXXII

Bound-Water Percentages (Method of Newton and Gortner, 1922) in the Expressed Saps from Winter Wheat Varieties (Data of Newton, 1923)

Variety. Plants from Field Plots	Total Solids, Per Cent	Bound Water, Per Cent	Variety. Plants from Field Plots	Total Solids, Per Cent	Bound Water, Per Cent
Hardy:			*Non-hardy:*		
Minhardi	16.4	14.4	Super	9.7	4.4
Buffum	17.8	13.0	Fulcaster	10.3	4.3
Semihardy:			*Plants from greenhouse:*		
Turkey	13.5	9.7	Minhardi	8.5	2.2
Kanred	13.5	8.1	Super	7.1	0.9
			Cactus (*Cereus sp.*)	4.9	8.3

In the summer of 1922, Harris[40] and his co-workers applied the technic of Newton and Gortner to drought resistance problems, utilizing

[40] Unpublished data.

as materials cereals which were growing under dry-land farming conditions and under irrigation in Utah. They found in general that when cereals have an abundant supply of moisture, there is relatively little tendency for the development of hydrophilic colloids and the elaboration of bound water, whereas when they are growing in conditions of stress, the onset of drought causes a greater proportion of the water in the tissues to be transformed from a free to a bound condition.

Newton, in Alberta, has continued the studies of bound and free water as related to drought resistance and has demonstrated quantitatively that drought resistance of agricultural crops and of native grasses is related to, or at least that drought resistant varieties can be differentiated by, bound water content. Tables XXXIII and XXXIV show certain of Newton's unpublished data. Table XXXIII is interesting because it affords a biochemical explanation of the reason as to why timothy almost invariably fails under the climatic conditions of western Alberta and why western rye grass will successfully grow under the Alberta moisture conditions. Timothy apparently does not possess the ability to bind water, whereas western rye grass does possess such ability.

TABLE XXXIII

Bound Water and Osmotic Pressure of Press-Juice of Timothy and Western Rye Grass Collected from the University of Alberta Experimental Plots in 1924 (Data of Newton and Co-Workers).

Species	Date of Collection	Osmotic Pressure, Atm.	Bound Water, Per Cent
Phleum pratense	July 16	11.6	1.4
	July 29	8.9	0.5
	August 22	8.8	0.5
Agropyron tenerum	July 29	12.5	6.3
	August 22	15.9	10.1

The technic of Newton and Gortner probably does not yield a true measure of the relationship between bound and free water as present in the cells and tissues of the plant. It is more probable that it yields minimum values for bound water. The method of determination presupposes (*a*) that bound water is not changed to free water by the freezing process, and (*b*) that the addition of sucrose does not alter the bound $\leftrightarrows$ free water equilibrium, and (c) that none of the water which is firmly associated with the hydrophilic colloids is free to dissolve sucrose. It seems highly probable from our knowledge of hydrophilic colloids that

TABLE XXXIV

Bound Water and Osmotic Pressure of Press-Juice of Grasses Collected at Edmonton, Alberta, 1925 (Data of Newton and Co-workers)

Species	Osmotic Pressure, Atm.	Bound Water, Per Cent	Habitat	Remarks
Phleum pratense (average of 7 collections)	15.71	3.12	Cultivated field	Moisture conditions fair.
Poa pratensis	13.36	4.66	Cultivated field	Moisture conditions fair.
Bechmannia erucaeformis	11.27	3.09	Slough	Plants standing in 2 inches of water.
Panicularia grandis	9.66	4.40	Slough	Soil saturated with water.
Fluminea festucaceae	13.42	4.29	Slough	Soil saturated with water.
Calamagrostis canadensis	11.87	3.71	Woodland	Moisture abundant.
Calamovilfa longifolia	13.21	6.91	Lower Slope of hillside	Moisture somewhat restricted at time of collection.
Agropyron tenerum (average of 7 collections)	18.68	10.34	Cultivated field	Moisture conditions fair.
Agropyron cristatum (average of 3 collections)	20.27	11.70	Cultivated field	Moisture conditions fair.
Agropyron Smithii (average of 3 collections)	17.37	6.42	Cultivated field	Moisture conditions fair.
Bromus inermis	19.72	10.60	Cultivated field	Moisture conditions fair.
Bouteloua gracilis	34.91	16.73	Dry hillside	Very dry soil.
Stipa comata	23.22	15.14	Dry hillside	Very dry soil.

Bound Water and Osmotic Pressure of Press-Juice of Cactus

Opuntia polycantha	7.57	16.61	Dry plains	Very dry soil.

the freezing process does shift the bound $\leftrightharpoons$ free water equilibrium toward the right, and in all probability the increased osmotic pressure due to the addition of sucrose will cause a further shift in the same direction. Accordingly the values obtained are probably minimum values and as such are more valuable than they would be if they were maximum values.

Thoenes[41] has proposed an entirely different technic for differentiating free and bound water. In his studies the hypothesis was made that bound water would not freeze at a temperature of $-20°$ C., whereas water in the liquid state would be frozen at that temperature. Accordingly Thoenes cools his samples for several hours to a temperature of approximately $-20°$ C. and then determines the proportion of the water which has crystallized in the form of ice by immersing the entire sample in water of known temperature in a calorimeter and observing the amount of heat which is necessary to thaw the ice and bring the entire mass to a temperature equilibrium. The latent heat of fusion of ice is 80 calories per gram. Knowing the specific heat of the solid material, it is possible to calculate how much of the water was in the form of ice. Thoenes proposes the formula,

$$X = \frac{C \cdot (T - T_1) - a \cdot s \cdot (T_1 + T_0)}{80 - \frac{T_0}{2}}, \tag{82}$$

where X = the quantity of water which has crystallized in the form of ice;
T_0 = the temperature of the gel at the time it is placed in the calorimeter (*e.g.*, $-20°$ C.);
T_1 = the equilibrium temperature of the water in the calorimeter;
T = the initial temperature of the water in the calorimeter;
a = the weight of the gel;
s = the specific heat of the gel;
C = the constant of the calorimeter system.

Knowing the initial moisture content of the gel, the difference between this value and (X) of equation (82) gives the quantity of water which is not frozen, or bound water. Table XXXV shows the bound water in certain substances, as determined by Thoenes.

Robinson[42] has applied Thoenes' methods to the study of winter

[41] Thoenes, F., Untersuchungen zur Frage der Wasserbindung in Kolloiden und tierischen Geweben, *Biochem. Z.*, 157 : 174–186 (1925).

[42] Robinson, W., Water Binding Capacity of Colloids, a Definite Factor in Winter Hardiness of Insects, *J. Econ. Entom.*, 20 : 80–88 (1927).

hardiness in insects, pointing out that the low temperatures of winter are, in many instances, a very effective means of natural control, functioning to reduce the number of injurious insects which emerge in the spring. In preliminary studies he used the winter-hardy *Telea polyphemus*, the moderately hardy *Callosamia promethea*, and the non-hardy granary weevil (*Sitophilus granarius*) as test material. The pupae of the first two species live over the winter within cocoons attached to the branches of shrubs upon which the larvae feed, and accordingly are exposed to the lowest temperatures of the winter in the regions where they occur. The granary weevil, on the other hand, is rarely exposed to temperatures below 5° C., and cannot exist if exposed for more than a few days to temperatures lower than approximately 2° C.

TABLE XXXV

Percentages of Bound and Free Water in Certain Animal and Plant Tissues as well as of Gelatin and Agar Gels (Data of Thoenes)

Material	Age	*p*H	Total Water, Per Cent	Free Water, Per Cent	Bound Water, Per Cent	Bound Water for Each Gram of Dry Matter, Gm.
Dog muscle	24 hours		85.7	59.0	26.7	1.86
Dog muscle	3 weeks		83.8	60.4	23.4	1.44
Dog muscle	4 weeks		83.3	59.7	23.6	1.40
Dog muscle	Several months		79.3	55.1	24.2	1.16
Dog muscle	Several months		82.0	62.9	19.1	1.06
Dog muscle	Several months		79.7	58.3	21.4	1.05
Guinea pig muscle	Young (160 gm.)		81.6	61.5	20.1	1.09
Guinea pig muscle	Old (600 gm.)		79.6	60.5	19.2	0.94
Laminaria		5.5	57.2	21.25	37.8	0.92
Laminaria		6.2	69.8	32.4	37.4	1.19
Laminaria		8.0	62.5	28.3	34.2	0.91
Gelatin		5.3	87.0	62.8	24.2	1.86
Geltain		4.3	86.4	60.25	26.2	1.92
Gelatin		3.0	87.1	59.5	27.6	2.14
Agar		5.5	94.1	69.55	24.55	4.15

Robinson found that the hardy *T. polyphemus* and the moderately hardy *C. promethea* showed evidences of a "hardening off" period exactly analogous to Newton's observations with winter wheat. The animal material in the non-hardy condition contained only 9 to 10 per

cent of bound water which progressively increased as both species were subjected to low temperatures until finally approximately 42 per cent of the moisture content of *C. promethea*, and 48 to 52 per cent of the moisture content of *T. polyphemus* was in the form of bound water. Figure 85, taken from the data of Robinson, shows the increase in percentage of bound water with the corresponding decrease in the percentage of free water as related to the temperature changes to which the material was subjected. The moisture content of the insects remained constant throughout the period of observation. In Robinson's experiments, the material was hardened off under rigidly controlled conditions, insofar as temperature and humidity were concerned. Material collected from out-of-doors, however, gave results exactly similar to the laboratory controlled material.

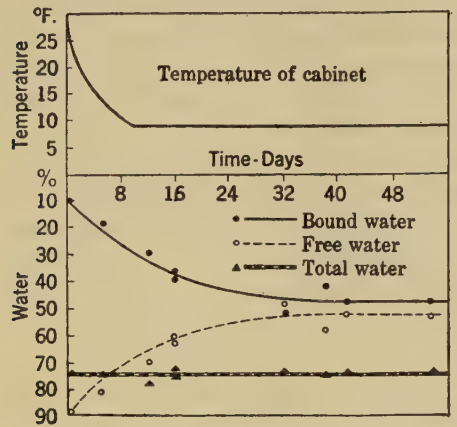


FIG. 85.—Curves showing total, free and bound water content of the pupae of *Callosamia promethea* in relation to temperature. (Data of Robinson.)

In the case of the granary weevil, exactly opposite observations were recorded, as shown in Fig. 86. About 35 per cent of the water content of these insects in their normal environment is in the form of bound water. Lowering the temperature causes a rapid change from bound to free water, the insect dying, as noted above, before the freezing point is reached. The granary weevil must be looked upon as a drought-resistant organism, living as it does in stored grain having a moisture content of 12 per cent or less. It is necessary for such an insect to conserve in some manner the amount of water in its body fluids, and this it

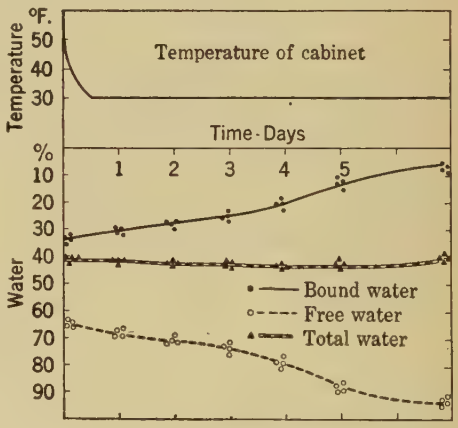


FIG. 86.—Curves showing the total, free and bound water of the granary weevil, *Sitophilus granarius*, in relation to temperature. (Data of Robinson.)

apparently does, utilizing more or less the same sort of mechanism as is utilized by the extreme xerophytes, binding the water on lyophilic colloids. A lowering of the temperature apparently destroyed the gel structure, converting the normal amount of bound water into free water.

In a later paper Robinson[43] discusses these problems at greater length, including the modified technic of Thoenes which he has found it necessary to adopt for his studies. The formula which he proposes is,

$$X = \frac{FN(T_3 - T_4) - SW(T_2 + T_4)}{80 - \frac{T_2 - T_1}{2}} \tag{83}$$

where X = the amount of free water within the material;
F = the correction factor for the calorimeter;
N = weight in grams of the water in the calorimeter;
T_3 = temperature of the water in the calorimeter at the beginning of the test;
T_4 = temperature of the water in the calorimeter at the end of the test;
W = weight of material in grams;
S = specific heat of the material;
T_2 = temperature of the material at the beginning of the test;
T_1 = the average freezing point of similar material.

Robinson points out that (T_2) is, of course, below zero and thus has a minus sign. He notes, however, that this sign should be disregarded and that (T_4) should be added to it and (T_1) subtracted from it as if it were plus.

In later work Robinson[44, 45] has shown that the amount of bound water in a series of insects is very closely correlated with the moisture content of the material upon which the insect feeds Thus, as already noted, the granary weevil under normal conditions has a high bound-water content. The granary weevil, feeding on extremely dry material, stands at one end of the series, while aphids, feeding upon succulent leaves, are at the other end of the series and possess under normal conditions practically no bound water. Robinson concludes from this series of experiments that the ratio between bound and free water in insects

[43] Robinson, W., Relation of Hydrophilic Colloids to Winter Hardiness of Insects, Colloid Symposium Monograph, Vol. V, pp. 199–218 (1928).

[44] Robinson, W., Response and Adaptation of Insects to External Stimuli, *Ann. Entomological Soc. of Am.*, 21 : 407–417 (1928).

[45] Robinson, W., Water Conservation in Insects, *J. Econ. Entomology*, 21 : 897–902 (1928).

regulates to a very large degree the rate of water loss from the insect. The granary weevil must have some mechanism which would prevent a rapid water loss and which probably even allows the insect to conserve the metabolic water produced by its own respiration. On the other hand, the aphids, drawing as they do extremely dilute juices from the plant, must evaporate large quantities of water from their body; otherwise the amount of nutrients which they could obtain would be be extremely small.

It would appear from these observations of Robinson, taken in connection with the work of Newton and of Thoenes, that the cell activities of both plant and animal organisms are to a very large measure regulated by a bound $\rightleftharpoons$ free water equilibrium which can, under certain conditions of stress, be shifted in one direction or another in order to provide for the preservation of the species.

Certain indirect evidence of a bound $\rightleftharpoons$ free water equilibrium is afforded by the data of Bailey,[46] the work of Whitcomb and Sharp,[47] and that of Jackson.[48]

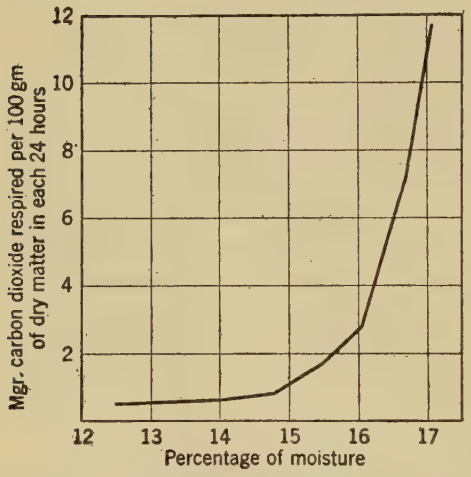


FIG. 87.—Respiration (heating) curve of wheat in relation to moisture content of grain. (Data of Bailey.)

Bailey studied the respiration of wheat kernels in relation to the moisture content of the grain. Figure 87 shows the relationship which he found. It will be noted that there is a sharp break in the respiration curve at about 14.75 per cent of moisture, and that an additional 1 per cent of moisture increases respiration nearly 200 per cent, and that a further 1 per cent of moisture increases it several hundred per cent more. It appears altogether probable that the sudden rise in respiration rate may be due to the fact that at, or below, 14.75 per cent moisture all, or

[46] Bailey, C. H., and Gurjar, A. M., Respiration of Stored Wheat, *J. Agr. Res.*, 12 : 685–713 (1918).

[47] Whitcomb, W. O., and Sharp, P. F., Germination of Frozen and Non-Frozen Wheat Harvested at Various Stages of Maturity, *J. Agr. Res.*, 31 : 1179–1188 (1925).

[48] Jackson, C. M., Storage of Water in Various Parts of the Earthworm at Different Stages of Exsiccation, *Proc. Soc. Exp. Biol. and Med.*, 23 : 500–504 (1925).

practically all, of the moisture in the wheat kernel is in the form of bound water, whereas free water is present in appreciable amounts whenever the percentage of moisture exceeds 14.75 per cent. Metabolic activity accordingly would be directly related to this increase in free moisture content, whereas dormancy would correspond to bound water content.

Whitcomb and Sharp studied the germination of wheat kernels which had been frozen at different stages of maturity. Certain of their data are recorded in Table XXXVI. It will be noted that kernels containing 56 per cent or more of moisture were almost uniformly killed by freezing, whereas kernels containing 50.6 per cent or less of moisture were highly resistant, the percentage germinating ranging from 60 to 98 per cent. Obviously a difference of 5.4 per cent of moisture in the kernel cannot account for a difference between 3 per cent germination and 72 per cent germination, and it appears very probable that a large part of the water represented by the 50.6 per cent moisture content is in a bound form.

Jackson studied the degree to which earthworms (*Lumbricus terrestris*) could be desiccated without loss of vitality. The earthworms were allowed to dry down at room temperature on filter paper, the loss in weight being followed by weighing at frequent intervals. Before the beginning of the experiment the earthworms had been kept for a day or two barely covered with tap-water until the weight reached a constant value. When desiccated to a point where they had lost not more than 43 per cent of their body weight, the organisms invariably recovered when returned to water. On the other hand, those which had lost 50 per cent of their body weight invariably failed to revive. There is a critical threshold below which water loss cannot go and the organism still recover, this threshold lying somewhere between 43 and 50 per cent loss of body weight. It seems altogether improbable that there would be such a sharp critical threshold if all the water in the organism were present in the same form, and these observations, taken in conjunction with those already noted, can be best interpreted in terms of a free $\rightleftharpoons$ bound water equilibrium where the free water content is exhausted at somewhere between 43 and 50 per cent of the body weight of the organism.

Coblentz[49] has indicated some very important observations which apparently bear upon the question of bound water. In a study of the form of water in minerals, Coblentz made use of infra-red adsorption spectra, and points out that water possesses pronounced infra-red adsorption bands at 1.5μ and 2.0μ. In the closing paragraphs of his

[49] Coblentz, W. F., The Role of Water in Minerals, *J. Franklin Institute*, 172 : 309–335 (1911).

TABLE XXXVI

Marquis Wheat: Germination of Frozen (Temperature −20° to −28° C.) and Non-frozen Wheat Harvested at Various Stages of Maturity* (Data of Whitcomb and Sharp, 1925).

Laboratory Number	Approximate Age of Kernel	Moisture at Time of Harvest	Weight per Kernel, Moisture-free	Germination			
				Nov. 24, 1923, Alternating Temperature 20° to 30° C., Nine Days	Dec. 21, 1923		Nov. 22, 1924, Alternating Temperature 20° to 30° C., Six Days
					Alternating Temperature 20° to 30° C., Six Days	Ice-box, Five Days; Alternating Temperature, Three Days	
	Days	Per Cent	Mg.	Per Cent	Per Cent	Per Cent	Per Cent
131, non-frozen	13	69.4	7.7	98	95	99	91
132, frozen	13	69.4	....	1	2	0	0
133, non-frozen	17	62.5	14.0	98	99	99	96
134, frozen	17	62.5	....	3	5	3	2
135, non-frozen	21	56.2	19.5	99	100	100	97
136, frozen	21	56.2	....	2	3	3	1
137, non-frozen	25	50.6	25.3	99	100	99	98
138, frozen	25	50.6	....	57	72	62	35
139, non-frozen	27	46.5	25.7	99	99	98	94
140, frozen	27	46.5	....	42	60	80	22
141, non-frozen	29	46.5	26.8	99	100	98	96
142, frozen	29	46.5	....	17	66	63	9
143, non-frozen	31	45.0	28.5	99	100	93	89
144, frozen	31	45.0	....	10	84	89	0
145, non-frozen†	33	43.5	29.9	98	99	98	98
146, frozen	33	43.5	....	42	77	85	27
147, non-frozen	35	38.7	30.7	99	100	100	93
148, frozen	35	38.7	....	42	80	80	32
149, non-frozen	38	34.1	30.2	100	100	99	95
150, frozen	38	34.1	....	72	89	88	63
151, non-frozen	41	....	31.0	99	100	99	98
152, frozen	41	....	....	92	98	97	82

*Harvest began Aug. 9, 1923, when the kernels were about thirteen days old. The germination tests were all run on duplicates of 100 kernels. Where 0.5 per cent occurred in the average it was added as 1 per cent. †Main part of field harvested.

paper he lists a single experiment with a gelatin film and notes that the general appearance of the infra-red transmission curve is entirely unlike that of water or of substances containing water, that the adsorption bands are sharp and narrow and resemble those characteristic of carbohydrates, adding, " In fact, the whole curve appears as though the water was present as ' water of constitution ' . . . . . This conclusion that the water in gelatin is constitutional instead of being held as is water in a sponge, is in agreement with the notions held by several chemists with whom the writer has discussed these data."

Insofar as the author is aware, this is the only observation dealing with infra-red adsorption spectra of lyophilic colloids. No data are given by Coblentz as to the percentage of water present in his " gelatin film." Neither are there any data as to the hydrogen ion concentration of the gelatin gel. It would be of extreme importance if this observation of Coblentz could be extended to include a series of gelatin films of varying hydrogen ion concentrations and varying moisture contents. It is possible that infra-red adsorption studies on such systems might afford another method of differentiating between free and bound water and might in fact yield maximum bound-water values in contrast to the methods already discussed which yield more or less minimum values.

Imbibition of Lyophilic Colloids in Normal Physiological Processes.—The possible role of imbibition in glandular secretion and in muscle contraction has already been referred to. In addition, we can consider that imbibition and syneresis play very important roles in the digestive and assimilative processes. In fact, osmotic phenomena within the living organism may be regarded, at least in part, as dependent upon imbibition forces.

The osmotic membranes or semi-permeable membranes of living organisms are invariably lyophilic gels. Much has been written in regard to the chemical nature of such membranes. Overton postulated that the plasma membrane is of a lipide nature, due to the fact that anaesthetics and fat-soluble materials pass readily into a cell, whereas salt solutions and substances which are typically water-soluble, as contrasted with lipide-soluble, pass with difficulty.

The author has taken the viewpoint that the plasma membrane is not necessarily a structure formed by vital forces but that a purely mechanistic explanation can be given for the formation of a plasma membrane. It would seem as though the formation of the plasma membrane was the inevitable consequence of the chemical composition of protoplasm. As already noted, protoplasm is composed, insofar as the organic portion is concerned, largely of proteins together with a certain proportion of fats and lipides. If a protein-fat-lipide mixture is allowed

to come in contact with water or a salt solution at an interface, an interfacial tension will develop, and *accordingly Gibbs' equation* (79) *will determine what compounds are present in the interface.* Those substances which decrease interfacial tension will be concentrated in the interface. Since lipides, such as lecithin, and the other phospholipides, as well as fats and the salts of the fatty acids markedly lower interfacial tension, these compounds will be concentrated in an interface, and consequently Overton would be correct in postulating a lipide layer. On the other hand, proteins are likewise efficient depressors of interfacial tension and on theoretical grounds it is impossible to picture an interface between protoplasm and either water or a salt solution where an increased concentration of proteins, as well as fats and lipides, is absent. Accordingly Overton must be only partly correct in his lipide theory, and *the plasma membrane, from a purely mechanistic standpoint, must contain any and all of the constituents of protoplasm which effect a lowering of the surface energy at an interface.* Such a mechanistic picture of the plasma membrane would probably consist of a more or less completely denatured (surface-energy coagulated) protein gel, probably in the form of a fibrillar structure with fats, soaps, and lipides immeshed in the protein network. The transfer of lipide-soluble materials would be through the fat-soap-lipide portion of the structure, whereas the passage of water and such water-soluble materials as actually do pass in and out of the cells would be through the hydrated filaments of the protein net-work.

If a semi-permeable membrane has the structure noted above, the transference of water across such a membrane, such as occurs in osmotic phenomena, can be considered to be due, at least in part, to an imbibition gradient across the membrane. Let us assume, for example, pure water on one side of such a membrane and a salt solution on the opposite side. Proteins have a lowered imbibition capacity when in contact with a salt solution. Accordingly we would expect the protein fibrils in contact with the salt solution to have a decreased water content, whereas those fibrils on the opposite side of the membrane in contact with pure water or with a solution of lower concentration, to have a higher water content. This would provide a hydration gradient across the fiber, and the normal process of diffusion in re-establishing an equilibrium within the protein fiber would cause a transfer of water from the point of lower salt concentration to the point of higher salt concentration.

A similar argument could be set up where non-electrolytes were present on one side of a membrane and pure water on the other side. Such a viewpoint attributes more than a passive role to the membrane, and while the ultimate equilibrium is determined by the relative concentrations on the two sides of the membrane, the membrane itself,

according to the above hypothesis, plays a definite role in osmotic phenomena.

The transfer of fluids from the digestive tract to the body tissues and the transfer of liquid from the body tissues to the digestive tract undoubtedly involve both imbibition and syneresis. A normal individual secretes from 700 to 1000 cc. of saliva per day, from 600 to 900 cc. of bile, from 600 to 800 cc. of pancreatic juice, from 1000 to 2000 cc. of gastric juice, whereas the water intake is usually only from 1000 to 1500 cc. Accordingly there passes into the digestive tract from 3.9 to 6.2 liters per day or from 4 to 6 times the amount of liquid that is taken in the form of liquid food, indicating that the water which is absorbed in the digestive tract is used several times over in the form of liquids which are secreted into the digestive tract. The passage of this relatively enormous volume of fluids from the digestive tract into the blood stream cannot be adequately accounted for by pure osmosis, since in many instances the contents of the digestive tract have a higher osmotic pressure than has the blood serum.

Imbibition, however, can account for this transfer of liquid. The proteins of the intestinal wall take up the water from the intestinal tract and transfer it to the proteins of the blood stream, which in turn carry it to other portions of the body where their imbibitional capacity is lowered and where they yield the water to other tissues or glands. The crystalloids which pass from the digestive tract to the blood stream and from the blood stream to the various cells and tissues of the body can be regarded as diffusing through a swollen gel rather than moving by osmotic processes across a membrane. Those chemicals which favor swelling of proteins, favor absorption from the intestinal tract, and those substances which hinder the swelling of proteins, hinder or prevent absorption from the intestinal tract. For example, magnesium sulfate more or less inhibits protein imbibition, and the effect of magnesium sulfate as a saline cathartic is to a large extent due to the prevention of the absorption of liquid from the intestinal tract, retaining the liquid within the tract. Similarly, agar, which furnishes bulk in cases of chronic constipation, is efficient because of its very pronounced hydrophilic nature, holding the water by imbibition forces against the pull of the intestinal wall and of the blood stream.

The Imbibition of Lyophilic Colloids as Related to Medical Problems.—It would be beyond the scope of this discussion to more than mention certain of the pathological problems which appear to be, at least in part, determined by lyophilic colloid-water relationships. The work of Martin Fischer on oedema and nephritis has already been casually referred to. Fischer has ably defended his viewpoint that oedema and

nephritis are diseases characterized by abnormal imbibition of the body colloids. He has presented an extremely strong case in the experiments which he has performed and in the case reports which he cites. Undoubtedly he is correct in the argument that in these diseases the affinities of the lyophilic colloids of the body for water are decidedly altered. Whether or not this alteration is the result of changes in hydrogen ion concentration or changes in salt concentration, or whether the observed results are due to some as yet unknown factors still is a problem for future research.

Fischer notes that in the normal organism the degree of imbibition of the tissues is very finely regulated. Thus, for example, the brain may swell as much as 1000 per cent if removed from the body and placed in various solutions. If, however, the brain were to swell 3 per cent in the living organism, the volume of the brain would become greater than the volume of the skull cavity, and the pressure of the brain on the skull cavity would produce at first intense pain, followed by death.

Fischer believes that the role of the salt content in the blood and tissues is at least in part the repression of the imbibition which would take place in the absence of such electrolytes. In this respect Bottazzi[50] suggests that the function of sodium chloride in the blood is to decrease the viscosity of the blood. If the inorganic salt content of the blood were decreased, as it sometimes is in the case of excessive perspiration, the hydration of the blood proteins would be increased, and accordingly the viscosity of the blood likewise would be increased.

This problem of excessive perspiration with the loss of relatively large quantities of sodium chloride from the body may have a rather important bearing on industry. Moss[51] notes that under muscular exertion and relatively high temperature, the sodium chloride content of perspiration ranges from 0.118 to 0.325 per cent, averaging 0.224, and that under such conditions more sodium chloride may be lost through perspiration than is lost in the urine. He suggests that workers in hot mines need more highly salted foods.

In certain of the very deep mines where the temperature regularly exceeds 100° F. a very considerable proportion of the miners developed intense headaches, necessitating hospital treatment at frequent intervals. In these mines the miners worked practically nude, drank large quantities of water, and due to the high temperatures and humidities were continuously bathed in perspiration. It occurred to the physician

[50] Bottazzi, F., Ricerche sopra soluzioni di colloidi organici, *Arch. fisiol.*, 7 : 579–637 (1909).

[51] Moss, K. N., Some Effects of High Air Temperatures and Muscular Exertion upon Colliers, *Proc. Roy. Soc.*, 95B : 181–200 (1923).

in charge that possibly the headaches might be attributed to an excessive loss of sodium chloride through the perspiration, causing an excessive imbibition of the body tissues, including the nervous tissue. Accordingly the recommendation was made that instead of the usual supply of water, the miners be furnished drinking water containing salts in approximately the proportion of a physiological salt solution. The effect of such substitution was striking in the extreme. The epidemic of headaches, which had persisted for years, disappeared as if by magic, indicating that the excessive loss of salts in the perspiration had been the determining factor.

Thomas and Andrews[52] have pointed out that blood serum from normal and nephritic individuals can be differentiated by the affinity of the serum proteins for water. The serum proteins from edematous individuals have a greater affinity for water than have the serum proteins from normal individuals. They note that normal sera generally do not swell at all and never swell more than 9 per cent, whereas sera from cases of uremia and oedema swell enormously, often exceeding 50 per cent. They suggest that these observations be made a method of diagnosis.

A similar method of diagnosis has recently come into use in certain hospitals, the technic being to inject small amounts of water intradermally, noting the length of time required for the absorption of the water by the tissues. An extremely rapid absorption is characteristic of edematous individuals or of individuals who will shortly develop oedemas, and such technic has permitted the detection of the onset of oedema considerably in advance of the time when it could be observed by the usual clinical methods.[53, 54]

The field of pathology offers a great opportunity to the colloid chemist who is interested in water relationships of the lyophilic colloids.

THE LIESEGANG PHENOMENA.—Liesegang[55] first described the peculiar reactions which take place when certain chemical processes are carried out in colloid gels. Thus, for example, if potassium chromate is dissolved in a gelatin gel and a solution of silver nitrate is allowed to

[52] Thomas, W. A., and Andrews, E., A New Dialysis Test for Tissue Thirst, *Proc. Soc. Exp. Biol. Med.*, 25 : 773–774 (1928).

[53] McClure, W. B., and Aldrich, C. A., Time Required for Disappearance of Intradermally Injected Salt Solution, *J. Am. Med. Assoc.*, 81 : 293–294 (1923).

[54] Aldrich, C. A., and McClure, W. B., The Intradermal Salt Solution Test. II. Its Prognostic Value in "Nephritis" with Generalized Edema, *J. Am. Med. Assoc.*, 82 : 1425–1428 (1924). Cf. also *J. Am. Med. Assoc.*, 83 : 1566–1567 (1924); 84 : 1258–1259 (1925); *Arch. Int. Med.*, 37 : 281–290 (1926); 41 : 102–211 (1928); *Surgery, Gynecology and Obstetrics*, July, 1926, pp. 40–45.

[55] Liesegang, R. E., Über einige Eigenschaften von Gallerten, *Naturwiss. Wochenschr.*, 11 : 353–362 (1896).

diffuse into the gel, the silver chromate which is formed is not dispersed uniformly throughout the gel but rather separates in a series of concentric rings, separated by more or less clear portions of the gel. Similarly, if potassium chromate is dissolved in sodium silicate and then the liquid

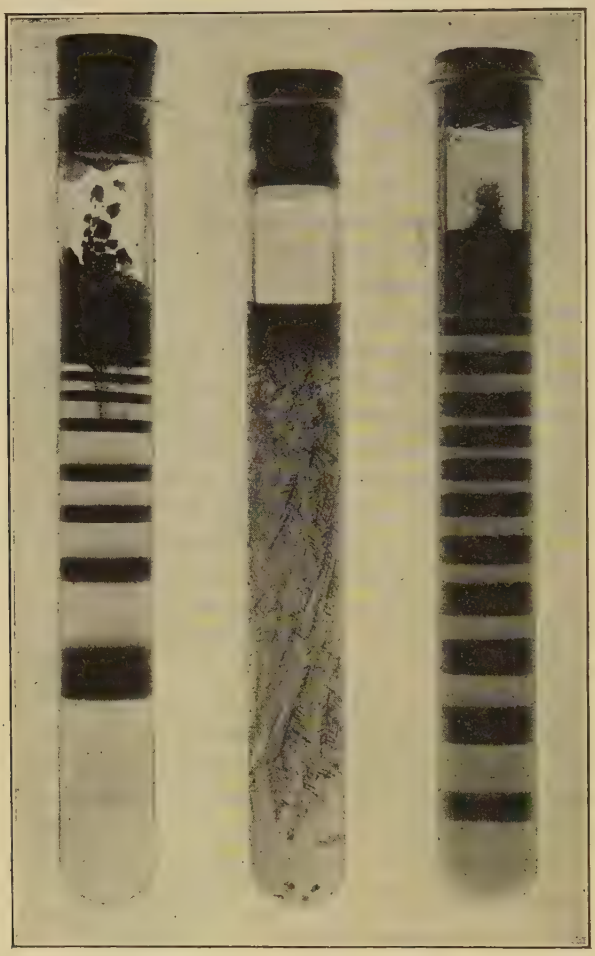


FIG. 88.—Liesegang phenomena. (A) and (C) copper chromate in silica gel; (B) lead iodide crystals in silica gel.

acidified so as to form a silicic acid gel, when copper sulfate diffuses into such a gel, banded precipitates of copper chromate will be produced. Such banded precipitates are known as Liesegang rings.

In other instances, for example, in the case of the reaction between potassium iodide and lead acetate, bands are not formed, but rather the

lead iodide slowly separates in the form of very large crystals. Figure 88 illustrates the formation of copper chromate rings in a silicic acid gel, and the large crystals of lead iodide, likewise in silicic acid gel.

Much has been written in regard to this phenomenon, and numerous theories have been proposed. It is extremely difficult to evaluate the theories, and we still lack exact data by which one or the other of the theories can be proven.

It has been suggested that the silver ions, diffusing into a chromate-gelatin gel, form silver chromate which remains in a super-saturated state, due to the protective action of gelatin. As the concentration of the silver increases, the silver chromate micelles are coagulated and crystallize out in the gel, forming a more or less impermeable layer. This silver chromate band would adsorb silver ions, retarding the diffusion of such ions through the gel, but would not adsorb nitrate ions which would pass through, possibly in the form of nitric acid, and such ions would advance ahead of the silver ions. As the silver chromate gel ages, it would break down, become coarser in structure, allow the silver ions to pass through, and eventually a new point of super-saturation with following precipitation would be reached, causing the formation of a new band.

Such a theory, however, does not explain the specificity of the gel structure. Banded precipitates will be formed in a number of instances in silicic acid gels which are not formed in gelatin gels. Similarly, banded precipitates are formed in other instances in gelatin gels, whereas no Liesegang rings are formed by the same reaction in silica gel. The nature of the gel in which the precipitation takes place plays an important role, and no theory as yet proposed accounts for the specificity of the gel.

It appears possible that the reaction may be due to differences in concentration of the reacting ions which bring about progressive peptization and coagulation. Thus, in Fig. 6 we have shown that silver bromide is held in colloidal dispersion by an excess of either silver or bromide ions, and that complete precipitation occurs only when the concentrations of silver and bromide ions are approximately equivalent. One could postulate, therefore, that in the diffusion of silver nitrate into a chromate gel, there would be for a time an excess of chromate ions, resulting in the peptization of the precipitate until eventually a point would be reached at which the chromate and silver ions are equivalent. At this point one would expect a precipitate would be formed, making a band of silver chromate. A repetition of this process would account for succeeding bands. Here again, however, the specificity of the gel is not explained.

Liesegang has suggested that the bands characteristic of agates are

due to the Liesegang phenomena. This appears to be the case. Figure 89 is the photograph of an artificial agate produced under laboratory conditions by allowing copper sulfate to diffuse into a silicic acid gel containing potassium ferrocyanide. The silicic acid gel was allowed to set in a collodion bag. The collodion bag was immersed in a dilute solution of copper sulfate and after several weeks was removed and the ball of gel cut in two. It will be noted that the laboratory product resembles exactly the natural agate. Bhatnagar and Mathur[56] have prepared such artificial agates and have slowly dried them under pres-

FIG. 89.—A photograph of an "artificial agate" formed by allowing copper sulfate to diffuse into a silica gel containing potassium ferrocyanide. An example of a Liesegang ring phenomenon.

sure, in this way obtaining preparations which have a hardness of approximately 5.0 on the mineralogical scale and which can be cut and polished so as to make them practically indistinguishable from natural agates.

It frequently happens that precipitates which are not in exact rings form in gels. Under such conditions it almost invariably happens that the figures which do form are very symmetrical, *i.e.*, a line drawn down the center of the figure will yield two halves which are mirror images of each other. This problem of symmetry of structures formed in gels

[56] Bhatnagar, S. S., and Mathur, K. K., Studien über Bandstrukturen. Die Synthese gebänderter Steine, *Koll. Z.*, 30 : 368–371 (1922).

indicates relationships which may be of biological importance. However, here again no adequate explanation to account for the production of symmetrical figures has been offered.

Whether or not the Liesegang phenomena play a role in living processes is still an open question. Liesegang has suggested that the coloration patterns on butterfly wings, the stripes which occur on elytra of beetles, etc., may be due to these phenomena. Such explanations appear probable but are still unproven. Many interesting and beautiful museum specimens may be prepared by causing precipitates to form in the various gels. For those who are interested in following the literature in this field certain references are noted.[57-65]

[57] Hatschek, E., The Liesegang Phenomenon, Second Report on Colloid Chemistry, pp. 21–26, Brit. Assoc. Adv. Sci., London (1918).

[58] Holmes, H. N., Experiments in Rhythmic Banding, *J. Am. Chem. Soc.*, 40 : 1187–1195 (1918).

[59] Davies, E. C. H., Liesegang Rings. III. The Effect of Light and Hydrogen-Ion Concentration on the Formation of Colloidal Gold in Silicic Acid Gel. Rhythmic Bands of Purple of Cassius, *J. Am. Chem. Soc.*, 45 : 2261–2268 (1923).

[60] Bradford, S. C., Adsorptive Stratification in Gels, *Biochem. J.*, 10 : 169–175 (1916).

[61] Bradford, S. C., Adsorptive Stratification in Gels, II., *Biochem. J.*, 11 : 14–20 (1917).

[62] Ganguly, P. B., Studies on the Formation of Liesegang Rings, *J. Phys. Chem.*, 31 : 481–495 (1927).

[63] Schleussner, C. A., Diffusionsvorgänge in Gelatine, II. Beiträge zur Kenntnis des Liesegang'schen Phänomens, *Koll. Z.*, 34 : 338–341 (1924).

[64] Handovsky, H., and du Bois-Reymond, E., Die Reaktion von Alkaloiden mit jodhaltiger Gelatine, *Koll. Z.*, 33 : 347–348 (1923).

[65] Bolam, T. R., and MacKenzie, M. R., The Influence of Lyophilic Colloids on the Precipitation of Insoluble Salts, Gelatin, and Silver Chromate, Pt. I and Pt. II, *Trans. Faraday Soc.*, 22 : 151–177 (1926).

CHAPTER IX

OSMOTIC PRESSURE AND ELECTRICAL CONDUCTIVITY

It is necessary to again diverge at this point from a discussion of the phenomena which are characteristic of truly colloidal systems and to consider briefly two properties of true solutions, *i.e.*, osmotic pressure and electrical conductivity.

Osmotic Pressure.—Diffusion and osmosis are of extreme importance in living processes. The passage of gases from the atmosphere to the plant or animal cells, or from one cell to another, the passage of ions and molecules from the soil solution into the root hairs of the plants and the transfer of these ions or molecules throughout the plant, or from animal cell to animal cell are all controlled by either diffusion or osmosis.

Diffusion in Gases.—If we bring two different gases into a vessel, taking precautions so as not to mix them, we will find after a time that there is a perfectly uniform mixture throughout the vessel. If the two gases are separated by a membrane permeable to both, they will pass through the membrane in both directions, so that at equilibrium the mixture will have the same composition on both sides of the membrane. This process is called diffusion, regardless of whether or not a septum divides the two components.

We can differentiate between two kinds of diffusion, (1) diffusion where gases are separated by a membrane in which neither gas is soluble, and (2) diffusion through a moist membrane, the gases being more or less soluble in the liquid filling the pores of the membrane.

In the first case, a membrane might consist, for example, of a sheet of porous porcelain. *The undissolved gases diffuse at a rate which is inversely proportional to the square root of the density of the gas, pressure and temperature being held constant.* For example, if hydrogen, with a density of 1, is on one side of such a membrane, and oxygen, with a density of 16, is on the other side of the membrane, the rates of diffusion will be in proportion to $\sqrt{1}$ and $\sqrt{16}$, oxygen, therefore, having approximately one-fourth of the rate of diffusion of hydrogen. Due to the fact that the rate of diffusion is unequal, it is possible to build up in such a system a pressure during the diffusion process. Thus, for example, if equal volumes of hydrogen and oxygen are placed on opposite sides of a

porous membrane, as in (*A*), after a definite period of time their distribution will be as at (*B*). It will be noted that in (*B*) there are $2\frac{1}{2}$ volumes of gas on one side of the membrane and $5\frac{1}{2}$ volumes on the opposite side. Consequently, a pressure gradient has been set up. At (*C*), however, representing equilibrium conditions, this pressure gradient has disappeared.

4 vols. H_2	4 vols. O_2	$\rightarrow$	2 vols. H_2 $\frac{1}{2}$ vol. O_2	2 vols. H_2 $3\frac{1}{2}$ vols. O_2	$\rightarrow$	2 vols. H_2 2 vols. O_2	2 vols. H_2 2 vols. O_2
A			*B*			*C*	
Initial State			Intermediate State			Equilibrium	

In the second case a moist membrane, in which the gases are soluble, separates the two gases, the densities of the gases play no part and the rate of diffusion is directly proportional to the coefficient of solubility of the gases in the solvent. Thus, for example, carbon dioxide, which has a density of 44, will pass through a moist membrane much more rapidly than will oxygen, due to the fact that carbon dioxide is appreciably soluble in water, whereas the reverse conditions would hold for diffusion through a dry membrane.

The above relationships are of great importance in living processes. For example, the passage of carbon dioxide from the atmosphere into the plant cells where photosynthesis takes place, is controlled by both types of diffusion. Diffusion through the stomatal openings is largely the diffusion of undissolved gases and is inversely proportional to the density of the gas. As soon, however, as the gas passes the stomatal opening, it comes in contact with the moist cell membranes, and its further passage into the cell and through the cells depends upon its solubility in the liquid phase. Gases, accordingly, move through cell walls and through protoplasm not in the gaseous state but in solution, and the greater the water content of the tissues or the membranes through which diffusion must take place, the more rapidly will diffusion occur.

Diffusion in Liquids.—The diffusion of molecularly dispersed material in liquid systems is approximately in accordance with the gas laws. Boyle's law states that *the volume of a fixed mass of a given gas at constant temperature is inversely proportional to the pressure.*

$$V = K\left(\frac{1}{P}\right) \tag{84}$$

Consequently,

$$V_1P_1 = V_2P_2 = K \tag{85}$$

which states that *the product of the simultaneous values of the pressure and the volume for a fixed mass of gas at constant temperature is constant.*

Avogadro's hypothesis states that *equal volumes of gases at the same temperature and pressure contain an equal number of molecules.*

Charles' law states that *the volume of a fixed mass of a gas at constant pressure is increased by* 1/273 *of its volume at* 0° *C. for each degree's rise in temperature.* Consequently we may write,

$$PV = NRT \tag{86}$$

or,

$$P = \frac{NRT}{V} \tag{87}$$

an equation which defines the pressure which will be exerted in a given system by a given mass of gas at a given temperature.

Solutions obey the gas laws approximately, providing that (*V*) *in equations* (86) *and* (87) *is evaluated as equal to the volume of the solvent and not the volume of the system.* Consequently, since a gram molecule of gas occupying a volume of one liter exerts a pressure of approximately 22.4 atmospheres, a gram molecule weight of solute in 1000 cc. of solvent will exert an *osmotic pressure* of approximately 22.4 atmospheres. Diffusion in liquids is proportional to the difference in concentration, and at equilibrium there would be a uniform concentration of the solute existing throughout the system.

The Measurement of Osmotic Pressure.—In studies involving the osmotic pressure of aqueous solutions it is important to distinguish between *weight molar solutions* (1 gram molecule of solute in 1000 grams of water) and *volume molar solutions* (1 gram molecule of solute in 1 liter of solution). In the former only will the correct values be approximated.

Various methods have been proposed for the measurement of osmotic pressure of aqueous solutions.

1. The Direct Method.—The direct measurement was first used by Pfeffer[1] and was later modified by Morse and coworkers.[2, 3] In this method a semi-permeable membrane is employed to separate the solution, being studied, from a pure water phase. A semi-permeable membrane is permeable only to water and not to the ions or molecules contained in

[1] Pfeffer, W., Osmotische Untersuchungen Studien zur Zellmechanik, Leipzig (1877).

[2] Morse, H. N., and Horn, D. W., The Preparation of Osmotic Membranes by Electrolysis, *Am. Chem. J.*, 26 : 80–86 (1901).

[3] Morse, H. N., and Frazer, J. C. W., The Preparation of Cells for the Measurement of High Osmotic Pressures, *Am. Chem. J.*, 28 : 1–23 (1902).

the solution under investigation. Morse and Frazer made use of a copper ferrocyanide gel precipitated in the pores of a porous porcelain cup. The gel which is used for a membrane must be solvated or wetted by the liquid of the solution. As already noted under gels, the transfer of water across such a membrane may in part be due to imbibitional forces. In Morse and Frazer's experiments the solution in question was placed inside of the prepared porcelain cup, the entire apparatus placed in contact with water, and the hydrostatic pressure which was reached by the diffusion of water through the membrane toward the more concentrated solution, measured on a mercury manometer.

The Earl of Berkeley[4] has modified this technic by placing the solvent inside the cell and applying pressure to the external solution so as to keep the volume constant. This modification provides for a more rapid measurement and also yields more accurate values, inasmuch as the solution is not diluted by the passage of liquid through the semi-permeable membrane.

2. **Indirect Methods.**—(*a*) *The Plasmolytic Method.*—Nägeli, and in 1884 De Vries,[5, 6] have studied the plasmolytic method as a means of measuring osmotic pressure in plant tissues. In this method the tissue is sectioned so as to be only a few cells in thickness, and these sections are then placed in a graded series of solutions of known concentration. Microscopic observations made at intervals show that in certain of the solutions, liquid is withdrawn from the plant cell, the protoplasmic contents shrinking away from the cell wall. Such an appearance indicates that the solution in which the cells are immersed is of a higher osmotic pressure than the solution within the plant cell. Such solutions are called *hypertonic* solutions. In other solutions it will be noted that the plant cell absorbs water, becomes larger, and may eventually burst. This indicates that water is being absorbed by the plant cell from a solution which has a lower osmotic pressure than the cell contents. Such solutions are *hypotonic.* Between these two extremes there will be found some concentration of the external solution which will neither withdraw water from the plant cell nor yield water to the plant cell. These solutions are stated to be *isotonic* or *isosmotic* with the contents of the cell.

Figure 90 shows the appearance of filaments of *Spirogyra* undergoing plasmolysis in a 0.35 M cane sugar solution. At (*A*) is seen a cell in

[4] Berkeley, Earl of, and Hartley, E. G. J., A Method of Measuring Directly High Osmotic Pressures, *Proc. Roy. Soc.*, London, 73 : 436–443 (1904).

[5] De Vries, Hugo, Eine Methode zur Analyse der Turgorkräfte, *Jahrb. f. wiss. Bot.*, 14 : 427–601 (1884).

[6] De Vries, Hugo, Zur plasmolytischen Methodik, *Bot. Z.*, 42 : 289–298 (1884).

which the protoplasmic contents are still approximately normal. In cell (*B*) the protoplasmic contents have begun to draw away from the cellulose wall, due to the transfer of liquid from the cell toward a hypertonic sugar solution. At cell (*C*) the protoplasmic mass has been so highly dehydrated as to contract into a rather small clot. Reference may be made to the paper of Miss Delf[7] for a consideration of the technic which must be employed in plasmolytic studies.

There are several objections to the use of the plasmolytic method. In the first place, the cell or cells which are observed may be abnormal and not representative of the plant as a whole, or they may have been

FIG. 90.—Plasmolysis of cells of spirogyra in 0.35 M sucrose solution. Cell A is essentially normal; the plasmolysis increases progressively from B → C → C′. (Photograph, courtesy of Dr. Lloyd.)

injured in the sectioning operation. Another objection is that the solution in which the cells are placed may have a toxic action on the cell or may have a coagulating or peptizing action, thus changing the permeability of the cell membrane. Certain solutions exert a toxic action in the cell or the cell membrane, so that the plasmolytic results which are obtained may not yield true osmotic pressure values. Solutions of potassium nitrate and solutions of cane sugar have been very generally employed in plasmolytic studies. Beck[8] draws the conclusion that cane

[7] Delf, E. M., Studies of Protoplasmic Permeability by Measurement of Rate of Shrinkage of Turgid Tissues. I. The Influence of Temperature on the Permeability of Protoplasm to Water, *Ann. Bot.*, 30 : 283–310 (1916).

[8] Beck, W. A., Cane Sugar and Potassium Nitrate as Plasmolysing Agents, *Protoplasma*, 1 : 15–72 (1926).

sugar gives correct results for osmotic pressure but that potassium nitrate solutions do not.

Osterhout distinguishes between true and false plasmolysis and notes that dilute solutions or even water may cause plasmolysis, such as would normally be brought about by hypertonic solutions. He attributes this false plasmolysis to the coagulation of protoplasm, due to the entrance of water and states that it is especially noticeable in studies dealing with marine plants.

Maximow[9] has suggested a macro-method for studying the osmotic pressure of plant roots and root hairs. Figure 91 illustrates the experiment which Maximow has devised. The plant, in which one wishes to study the osmotic pressure of the root system, is grown in soil, and when it has reached the proper stage of growth, the soil is carefully washed away from the roots, care being taken not to mechanically injure the root structures. The entire plant is then immersed under water and the top cut off with a sharp knife. Connection is made between the cut portion of the stem and a bent glass tube by means of rubber tubing. The plant is then removed, and the roots placed in solutions of sucrose of varying concentration. When the roots are placed in hypotonic solutions, water is drawn through the plant cell walls by osmosis and liquid flows from the end of the glass tubing, as shown in Fig. 91. If, now, the plant is removed to a hypertonic solution, liquid is withdrawn from the root cells, and liquid not only ceases to flow from the tip of the glass tube, but actually recedes in the glass capillary. When the plant is placed in isotonic solutions, the liquid level remains stationary in the glass tube.

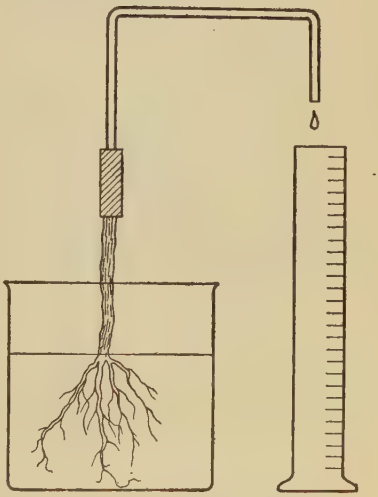


FIG. 91.—A diagrammatic representation of Maximow's method for determining the osmotic pressure of plant root cells. (In this instance, since there is an upward flow of liquid, the roots are in a hypotonic solution.)

Such a technic appears to afford a more accurate index of the osmotic pressure of roots than would the usual plasmolytic technic. There is little danger of mechanically injuring the roots, and the osmotic pressure obtained by the Maximow technic is the average osmotic pressure

[9] Maximow, N. A., Lecture on some phases of plant physiology, delivered at the University of Minnesota, October 26, 1926.

of the cell contents of the entire mass of roots, as determined under conditions very similar to the normal relations existing between a plant and the soil solution.

(*b*) *Vapor Pressure Methods.*—The vapor pressure methods of studying osmotic pressure depend upon the phenomenon of isothermal distillation of solutions of lower concentrations to those of higher concentrations. In Fig. 92 is shown a closed system with water in the lower portion of the container and a tube of sugar solution suspended in the system, the top of the tube being open, the bottom being closed with a semi-permeable membrane. Water rises in the tube containing the sugar to

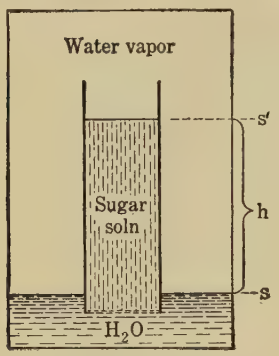


Fig. 92.

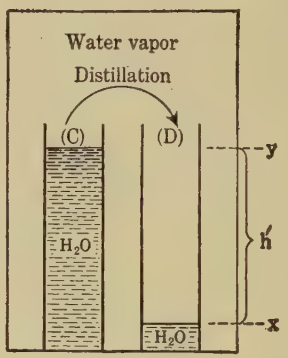


Fig. 93.

Fig. 92.—A diagrammatic representation of the relationship between vapor pressure and osmotic pressure.

Fig. 93.—A diagrammatic representation of isothermal distillation from a water surface at a higher level to one at a lower level.

the equilibrium point (s'), due to osmotic pressure. At this point the hydrostatic pressure of the solution in the tube which tends to drive the liquid downward through the semi-permeable membrane, just balances the osmotic pressure of the sugar solution, *i.e.*, the force which tends to draw water up through the membrane. The vapor pressure of the pure solvent must accordingly be greater than that of the sugar solution by an amount equal to the pressure of a column of water vapor of height (h) which is the difference in level between the two surfaces (s) and (s'). Therefore,

$$\text{Vapor pressure at } s = (\text{vapor pressure at } s') + h \qquad (88)$$

Only under such conditions will an equilibrium be reached and no distillation of water vapor take place.

If we were to construct a closed system containing two vessels, each containing water the surface of which was at the different levels, (x) and (y), as shown in Fig. 93, at *equilibrium* both vessels would contain water

at the same level, *i.e.*, there would be isothermal distillation from the water in (*C*) to the water in (*D*) because the vapor pressure of the water in (*C*) at the surface of the water is greater than that in the vapor phase above (*D*) at a corresponding level in the closed system by an amount which is proportional to the weight of a column of water vapor of height (*h'*). Of course, the *actual rate* of distillation would be very slow and might not be realizable experimentally within the usual time limits. The crucial point is that the system, shown in Fig. 93, is not in equilibrium.

In Fig. 94 we again have a closed system, inclosing two vessels, one containing pure water, the other a sugar solution. Here also distillation will be from the water to the sugar solution, and at equilibrium the beaker containing the water would be empty, all the water having been transferred to the sugar solution. The water vapor phase here acts as a semi-permeable membrane, permeable in one direction to water vapor but not permeable in the other direction to the sugar molecules. Hence, there is a distillation from the point of high vapor pressure to that of lower vapor pressure.

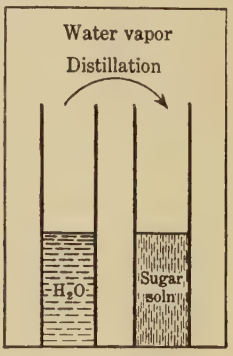


FIG. 94. — A diagrammatic representation of isothermal distillation from a solution of low osmotic pressure to one having a higher osmotic pressure.

Barger,[10] in 1904, used this method to determine the molecular weights of small quantities of organic compounds. Since the lowering of the vapor pressure is directly proportional to the osmotic pressure, which in turn is proportional to the molecular concentration, the extent to which equal weights of organic compounds will lower the vapor pressure of a solution is directly proportional to the molecular weight of the organic compound.

Halket[11] used this method to determine the osmotic pressure of plant saps. In her experiments only small quantities of plant saps were available. She prepared capillary glass tubes of very fine bore and cleaned them so scrupulously that the sap or liquids would not adhere to the glass wall. She then drew a small quantity of the plant sap into the capillary tube, followed by a tiny bubble of air, then by a drop of sugar solution of known concentration; this in turn was followed by another bubble of air, a drop of sap, a bubble of air, a drop of stronger sugar solution, etc., etc. This arrangement placed the drops of plant

[10] Barger, G., A Microscopical Method of Determining Molecular Weights, *J. Chem. Soc.*, London, 85 : 286–324 (1904).

[11] Halket, A. C., On Various Methods for Determining Osmotic Pressures, *New Phytologist*, 12 : 164–176 (1913).

sap between drops of sugar solutions of progressively increasing concentration. *A sugar solution with an osmotic pressure greater than that of the sap caused distillation from the sap to the sugar solution, so that the volume of the plant sap droplet decreased. A sugar solution with an osmotic pressure lower than that of the sap caused distillation from the sugar solution to the plant sap, and the volume of the sap droplet increased. That droplet that was so situated as to remain practically constant in size was judged to be placed between two sugar solutions, one of which had a slightly greater osmotic pressure and one a slightly lower osmotic pressure than had the plant sap.* In this way the osmotic pressure of very small quantities of solutions can be approximately determined. The increase or decrease in the volume of the droplets was measured by means of a micrometer eye-piece in a microscope.

(*c*) *Elevation of the Boiling Point.*—If the vapor pressure is lowered with increasing concentration of solute, it must be self-evident that the boiling point must be raised. Accordingly, osmotic pressure may be calculated by the increase in the boiling point. The technic for this determination can be found in any good text on physical chemistry. It will not be discussed in any detail here, due to the fact that biological systems are so readily altered at high temperatures as to render this method of little use when dealing with biological fluids.

(*d*) *Depression of the Freezing Point.*—Similarly, if there is an elevation of the boiling point and a decrease in vapor pressure due to an increased concentration of solute, solutions must freeze at a lower temperature than the pure solvent. Any adequate text on physical chemistry will afford directions for carrying out freezing point determinations.

The depression of the freezing point is one of the most generally used methods available to the biologist for the study of osmotic pressures of biological fluids. In dealing with plant saps, Dixon and Atkins[12] pointed out in 1913 that representative samples of plant saps could not be obtained by simply pressing the fresh tissues to extract the sap, inasmuch as the cell walls acted as ultrafilters, retaining a greater or less proportion of the solutes in the sap.

Gortner, Lawrence, and Harris[13] have confirmed the observations of Dixon and Atkins and have shown that when plant leaves are pressed without a preliminary freezing, saps may in some instances be obtained which are more dilute than the saps within the cells, or in other instances

[12] Dixon, H. H., and Atkins, W. R. G., Osmotic Pressures in Plants. I. Methods of Extracting Sap from Plant Organs, *Sci. Proc. Roy. Dublin Soc.* (n.s.) 13 : 422–433 (1913). Also, *Notes from Bot. Sch. Trinity Coll. Dublin*, 2 : 154–172 (1913).

[13] Gortner, R. A., Lawrence, J. V., and Harris, J. A., The Extraction of Sap from Plant Tissues by Pressure, *Biochem. Bull.*, 5 : 139–142 (1916).

more concentrated than such saps. Dixon and Atkins accordingly suggest freezing the tissues in liquid air and then thawing them, thus making the cell membranes permeable so that uniform and representative samples of cell sap could be obtained. Gortner and Harris[14] found that a preliminary freezing in an ice and salt mixture answered the same purpose. In this paper they likewise discussed the details of the method for the determination of the osmotic pressure of plant saps. Later Harris and Gortner[15] tabulated the values for the depression of the freezing point in terms of osmotic pressure, using the formula,

$$P = 12.06\Delta - 0.021\Delta^2 \tag{89}$$

where P = osmotic pressure;
Δ = *corrected* depression of the freezing point.

It is necessary to correct the observed depression of the freezing point in order to obtain the true depression. This is due to the fact that on freezing a certain amount of the water separates as ice. Accordingly, the system is then that of a more saturated solution than the original sap in equilibrium with ice crystals, and the observed depression of the freezing point is the freezing point of this more saturated solution. If a liter of pure water is under-cooled to -1° C. before ice crystallization begins, 1/80 of the water will separate in the form of ice, and when 1/80 has separated in the form of ice, the temperature of the liter of water will have risen to 0° C. This is due to the fact that the latent heat of fusion of ice is 80 calories. Consequently the crystallization of 1 gram of ice liberates 80 calories of heat. Using this value, one can easily correct the observed depression of the freezing point by means of the formula,

$$\Delta = \frac{\Delta'\left(V - \frac{uV}{80}\right)}{V} \tag{90}$$

or

$$\Delta = \Delta' - 0.0125\, u\Delta' \tag{91}$$

where Δ = the corrected depression of the freezing point;
Δ' = the observed depression of the freezing point;
u = degrees of under-cooling before ice separation begins.

[14] Gortner, R. A., and Harris, J. A., Notes on the Technique of the Determination of the Depression of the Freezing Point of Vegetable Saps, *Plant World*, 17 : 49–53 (1914).

[15] Harris, J. A., and Gortner, R. A., Notes on the Calculation of the Osmotic Pressure of Expressed Vegetable Saps from the Depression of the Freezing Point, with a Table for the Values of P for $\Delta = 0.001^\circ$ to $\Delta = 2.999^\circ$, *Am. J. Bot.*, 1 : 75–78 (1914).

Harris and Gortner recommend the use of a Haidenhain thermometer, fixed to read from $+1°$ to $-5°$ C. in $1/100°$ subdivisions, for the ordinary freezing point determinations of aqueous solutions.

Wright and Harvey[16] suggest the replacement of a thermometer by thermocouples.

Harris and his co-workers[17-28] have made extensive use of the method of the depression of the freezing point in studies of plant distribution as determined by environmental conditions, and have shown that valuable ecological data may be secured from such studies. In 1920, they found in a Utah alkali spot a plant, *Atriplex nuttallii*, the sap of which had a depression of the freezing point of 14.4° C., corresponding to an osmotic pressure of approximately 173 atmospheres. In later (unpublished) work Harris observed a slightly greater depression than this in the sap of *Atriplex confertifolia*.

As already noted in Chapter VIII, the studies of the depression of

[16] Wright, R. C., and Harvey R. B., The Freezing Point of Potatoes as Determined by the Thermoelectric Method, U. S. Dept. Agr. Bull. No. 895 (1921).

[17] Harris, J. A., Lawrence, J. V., and Gortner, R. A., On the Osmotic Pressure of the Juices of Desert Plants, *Science*, 41 : 656–658 (1915).

[18] Harris, J. A., Lawrence, J. V., and Gortner, R. A., The Cryoscopic Constants of Expressed Vegetable Saps, as Related to Local Environmental Conditions in the Arizona Deserts, *Physiol. Res.*, 2 : 1–49 (1916).

[19] Harris, J. A., and Lawrence, J. V., On the Osmotic Pressure of the Tissue Fluids of Jamaican Loranthaceae Parasitic on Various Hosts, *Am. J. Bot.*, 3 : 438–455 (1916).

[20] Harris, J. A., Physical Chemistry in the Service of Phyto-Geography, *Science*, 46 : 25–30 (1917).

[21] Harris, J. A., and Lawrence, J. V., Cryoscopic Determinations on Tissue Fluids of Plants of the Jamaican Coastal Deserts, *Bot. Gaz.*, 64 : 285–305 (1917).

[22] Harris, J. A., and Lawrence, J. V., The Osmotic Concentration of the Sap of the Leaves of Mangrove Trees, *Biol. Bull.*, 32 : 202–211 (1917).

[23] Harris, J. A., and Lawrence, J. V., The Osmotic Concentration of the Tissue Fluids of Jamaican Montane Rain-Forest Vegetation, *Am. J. Bot.*, 4 : 268–298 (1917).

[24] Harris, J. A., On the Osmotic Concentration of the Tissue Fluids of Phanerogamic Epiphytes, *Am. J. Bot.*, 5 : 490–506 (1918).

[25] Harris, J. A., On the Osmotic Concentration of the Tissue Fluids of Desert Loranthaceae, *Mem. Torrey Bot. Club.*, 17 : 307–315 (1918).

[26] Harris, J. A., Gortner, R. A., and Lawrence, J. V., The Osmotic Concentration and Electrical Conductivity of the Tissue Fluids of Ligneous and Herbaceous Plants, *J. Phys. Chem.*, 25 : 122–146 (1921).

[27] Harris, J. A., Lawrence, Z. W., Hoffman, W. F., Lawrence, J. V., and Valentine, A. T., The Tissue Fluids of Egyptian and Upland Cottons and Their F_1 Hybrid, *J. Agr. Res.*, 27 : 267–328 (1924).

[28] Harris, J. A., Gortner, R. A., Hoffman, W. F., Lawrence, J. V., and Valentine, A. T., The Osmotic Concentration, Specific Electrical Conductivity, and Chlorid Content of the Tissue Fluids of the Indicator Plants of Tooele Valley, Utah, *J. Agr. Res.*, 27 : 893–924 (1924).

the freezing point have been utilized by Newton and others in a technic for the determination of bound water in the presence of hydrophilic colloids.

Electrical Conductivity

The determination of the osmotic pressure of solutions or of biological fluids yields information which can be correlated with the actual concentration of molecularly dispersed substances present in such solutions, but gives no information as to the relative proportion of electrolytes and non-electrolytes.

According to the theory of Arrhenius, electrolytes in aqueous solutions are more or less completely dissociated into ions, and the conductance of an electric current through a solution of an electrolyte is due to the presence of these ions.

Using osmotic pressure technic, each ion will contribute independently to the osmotic pressure of the system. Thus, for example, assuming complete dissociation of sodium chloride, one gram molecule of sodium chloride would produce an osmotic pressure twice as great as one gram molecule of sucrose. The degree of ionization (or the " activity " of the ions) of electrolytes can be determined by making use of osmotic pressure technic.

In dealing with biological fluids and tissues it is often desirable to differentiate between the total solutes in a system and the relative concentration of electrolytes and non-electrolytes. Such a differentiation can be secured, at least in part, by studying the electrical conductivity of such fluids or tissues, and such studies have been used extensively in biological investigations, particularly as a means of detecting changes in permeability of protoplasm or of the plasma membrane.

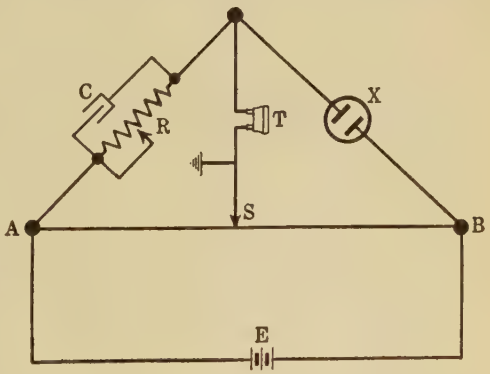


Fig. 95.—A diagrammatic representation of the conventional Wheatstone bridge for measuring electrical conductivity.

Figure 95 represents diagrammatically the apparatus necessary for the measurement of electrical conductivity. We have at (R) a variable resistance across which is shunted a variable air condenser (C), in order to balance the capacity of the system. (AB) is the slide wire of the

Wheatstone bridge, having uniform diameter and electrical resistance. (X) is the electrolytic cell, containing platinized electrodes between which is inserted the tissue or fluid under investigation. A source of electric current (E) is connected to the two ends of the bridge, and an electric current detecting apparatus, such as a telephone or a galvanometer, is connected, as shown at (T), to a sliding contact (S) attached to the resistance wire of the Wheatstone bridge. In such a set-up, when an electric current is impressed upon the system, the sliding contact is adjusted so that the current passing through the cell is exactly balanced by the variable resistance. The resistance of the material in the cell can then be calculated from the known value used at (R) and the ratio of the two arms of the Wheatstone bridge.

For biological tissues and solutions it is desirable to use as a source of electric current an alternating current of rather high frequency and purity. Currents of 1000 cycles are generally employed, although recently currents of radio frequency up to 5,000,000 cycles per second are coming more and more into use. When such currents are employed, it is, of course, necessary to replace the current detector by radio apparatus.

Measurements of electrolytic conductivity can be made rapidly and relatively accurately. In general, the values are expressed in terms of specific electrical conductivity, conductivity being the reciprocal of resistance. Resistance is measured in ohms. Conductivity is expressed in mhos.

$$\frac{1}{\text{ohms resistance}} = \text{mhos conductivity} \qquad (92)$$

Anything which impedes an ion in its passage through a solution or tissue increases the resistance of that system, and accordingly decreases the conductivity.

The specific conductivity is the conductivity of a cube of solution, one centimeter on an edge, between electrodes suspended exactly one centimeter apart, each electrode having one square centimeter of area. It is obvious that the construction of a conductivity cell having exactly these specifications would be extremely difficult. As a matter of fact, such construction is rarely attempted. Instead the "*cell constant*" of the cell is determined by using in the cell a solution, the specific electrical conductivity of which has already been determined, and from such data the cell constant is calculated from the formula,

$$\frac{B}{A} R_K = \text{cell constant} \qquad (93)$$

where B = the length of the arm of the bridge between (B) and (S) in Fig. 95;

A = the length of the arm of the bridge between (A) and (S) in Fig. 95;

R = the resistance in ohms, as measured on the resistance box;

κ = the specific electrical conductivity of the known solution.

TABLE XXXVII

Specific Conductivity of Standard KCl Solutions for Determining the Resistance-Capacity of Conductivity Vessels

Temperature	Concentration of KCl Solution			
	$\frac{N}{1}$	$\frac{N}{10}$	$\frac{N}{50}$	$\frac{N}{100}$
° C.	κ	κ	κ	κ
0	0.06541	0.00715	0.001521	0.000776
1	0.06713	0.00736	0.001566	0.000800
2	0.06886	0.00757	0.001612	0.000824
3	0.07061	0.00779	0.001659	0.000848
4	0.07237	0.00800	0.001705	0.000872
5	0.07414	0.00822	0.001752	0.000896
6	0.07593	0.00844	0.001800	0.000921
7	0.07773	0.00866	0.001848	0.000945
8	0.07954	0.00888	0.001896	0.000970
9	0.08136	0.00911	0.001945	0.000995
10	0.08319	0.00933	0.001994	0.001020
11	0.08504	0.00956	0.002043	0.001045
12	0.08689	0.00979	0.002093	0.001070
13	0.08876	0.01002	0.002142	0.001095
14	0.09063	0.01025	0.002193	0.001121
15	0.09252	0.01048	0.002243	0.001147
16	0.09441	0.01072	0.002294	0.001173
17	0.09631	0.01095	0.002345	0.001199
18	0.09822	0.01119	0.002397	0.001225
19	0.10014	0.01143	0.002449	0.001251
20	0.10207	0.01167	0.002501	0.001278
21	0.10400	0.01191	0.002553	0.001305
22	0.10594	0.01215	0.002606	0.001332
23	0.10789	0.01239	0.002659	0.001359
24	0.10984	0.01264	0.002712	0.001386
25	0.11180	0.01288	0.002765	0.001413
26	0.11377	0.01313	0.002819	0.001441
27	0.11574	0.01337	0.002873	0.001468
28	.....	0.01362	0.002927	0.001496
29	.....	0.01387	0.002981	0.001524
30	.....	0.01412	0.003036	0.001552

Generally, solutions of potassium chloride are used as standard solutions. Table XXXVII lists the specific electrical conductivity of various solutions of potassium chloride at various temperatures.

In dealing with biological fluids or tissues, or even in dealing with mixtures of electrolytes, it is impossible to translate specific electrical conductivity into concentration values. Accordingly, one cannot calculate from electrical conductivity determinations the concentration of

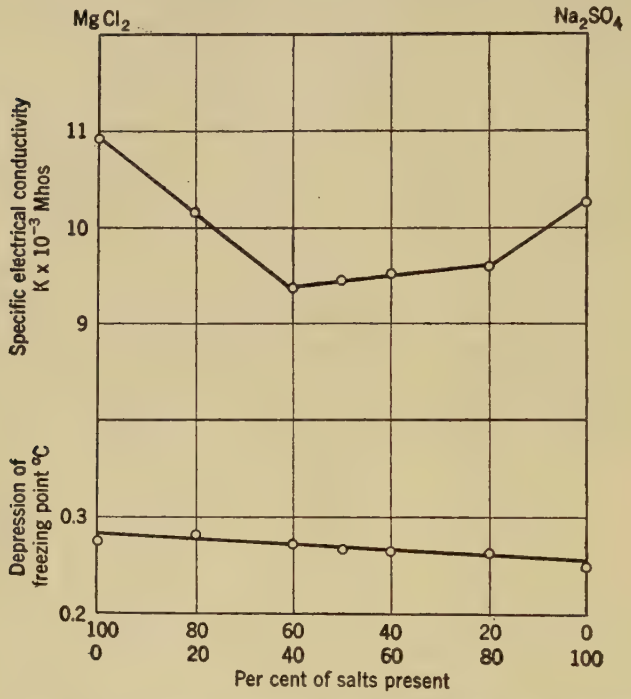


FIG. 96.—Showing the specific electrical conductivity and the depression of the freezing point of pure and mixed 0.1 N solutions of $MgCl_2$ and Na_2SO_4. (Data of Pascoe.)

non-electrolytes which are present in a given biological system. As a rule, osmotic pressure determinations are a more accurate index of the actual concentration of electrolytes and non-electrolytes than is electrical conductivity an estimation of the electrolyte content.

Harris, Gortner, and Lawrence[29] have suggested the use of the ratio,

[29] Harris, J. A., Gortner, R. A., and Lawrence, J. V., Studies on the Physico-chemical Properties of Vegetable Saps. 3. A Comparison of the Physico-chemical Constants of the Juices Expressed from the Wall with Those from the Included Carpellary Whorl in Proliferous Fruits of Passiflora gracilis, *Biochem. Bull.*, 4 : 52–79 (1915).

$\frac{\kappa}{\Delta}$, as of value in indicating changes in the ratio of electrolytes to non-electrolytes in plant saps, and this ratio has been used rather generally in the physico-chemical studies of plant saps as related to geographical environment, as noted in the references above.

In an attempt to ascertain whether or not mixtures of electrolytes have a constant $\frac{\kappa}{\Delta}$ ratio, Pascoe[30] studied the osmotic pressure (as

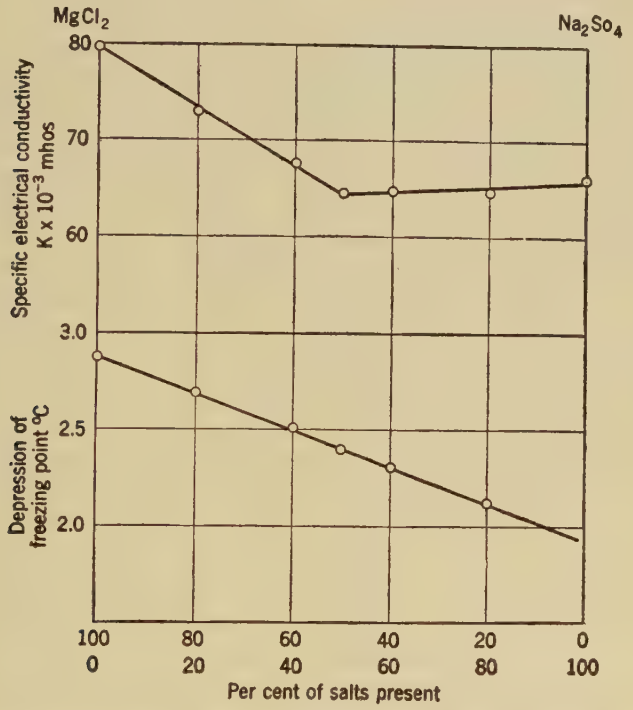


FIG. 97.—Showing the specific electrical conductivity and the depression of the freezing point of pure and mixed 1.0 N solutions of $MgCl_2$ and Na_2SO_4. (Data of Pascoe.)

determined by depression of the freezing point) and the specific electrical conductivity of a series of mixtures of salt solutions, using as electrolyte mixtures the systems, $MgCl_2$, $MgSO_4$; $MgCl_2$, Na_2SO_4; $MgCl_2$, NaCl; NaCl, Na_2SO_4; $MgSO_4$, Na_2SO_4; and NaCl, $MgSO_4$. Figures 96 and 97 show that the depression of the freezing point in mixtures of $MgCl_2$ and $MgSO_4$ is a linear function of the mixture, whereas the specific elec-

[30] Pascoe, T. A., Some Physico-Chemical Properties of Salt Solutions, Master's thesis, manuscript copy filed in Library of the University of Minnesota (1926).

trical conductivity curves in these figures indicate very clearly that electrical conductivity is not necessarily a linear function in mixed solutions. No physico-chemical theory appears to account for the peculiar forms of the conductivity curves. Similarly, no satisfactory theory of mixed salt solutions has yet been developed by the physical chemists.

The solutions which Pascoe used are more concentrated than solutions usually studied in physico-chemical laboratories, the justification for such concentrations being that biological reactions are, as a rule, carried out in the presence of relatively high concentrations of electrolytes. It is not unusual to find plant saps, at least in certain halophytes, which contain inorganic chlorides exceeding the concentration of 100 grams of sodium chloride per liter (calculated as sodium chloride from the chloride content of the plant sap). Similarly, biological reactions always occur in solutions of mixed electrolytes. The elucidation of the physico-chemical properties of such systems accordingly becomes of paramount interest to the biologist and to the biological chemist.

Changes in the hydrogen ion concentration of biological fluids, as a rule, do not make for a corresponding change in electrical conductivity. This is particularly true in physico-chemical studies of plant saps. The explanation probably lies in the fact that organic acids are relatively slightly ionized. Thus, for example, the electrical conductivity of the sap of the leaves of the gooseberry is very much greater than is the electrical conductivity of the fluids which can be expressed from the green berries. In general, leaf tissue fluids have a much higher conductivity than do fluids which can be expressed from either green or ripe fruits.

Harris, Gortner, and Lawrence,[31] in a study of the relation between the osmotic concentration of the leaf sap and the height of leaf insertion in trees, noted that the osmotic pressure, as measured by depression of the freezing point, increased with the tree height at which the leaves were collected, whereas the specific electrical conductivity decreased from the lower to the higher levels. A part of their data is shown in Table XXXVIII. This paper opens interesting possibilities. Evidently there is an increased photosynthesis in those leaves in the upper portion of a tree, as indicated by an increased osmotic pressure, and a decreased ratio of $\frac{\kappa}{\Delta}$. Whether or not this increase is due to more favorable light conditions, or whether it is due to a more favorable ratio of the electrolytes present in the cell sap can only be determined by more

[31] Harris, J. A., Gortner, R. A., and Lawrence, J. V., The Relationship between the Osmotic Concentration of Leaf Sap and Height of Leaf Insertion in Trees, *Bull. Torrey Bot. Club*, 44 : 267–286 (1917).

TABLE XXXVIII

Showing the Depression of the Freezing Point (Δ), the Osmotic Pressure in Atmospheres (P), the Specific Electrical Conductivity (κ), and the Ratio of Specific Conductivity to Depression of the Freezing Point $\left(\frac{\kappa}{\Delta}\right)$ for the Tissue Fluids of Leaves at Different Heights of Leaf Insertion in Trees.

Tree	Height	Δ	P	$\kappa \times 10^{-5}$	$\frac{\kappa}{\Delta} \times 10^{-5}$
	(Ft.)	(° C.)	(Atm.)	(Mhos)	
Acer rubrum.....	12	1.334	16.05	938	703
	27	1.359	16.35	911	671
	47	1.385	16.66	863	623
Juglans cinerea.....	8	1.398	16.81	1332	954
	21	1.484	17.85	1197	807
	32	1.513	18.19	1174	777
	38	1.429	17.18	1218	852
	44	1.525	18.33	1114	731
	52	1.522	18.31	1046	687
Quercus palustris.....	9	1.681	20.21	1057	629
	23	1.728	20.77	1002	580
	33	1.932	23.22	903	467
Robinia pseudacacia...	24	0.915	11.01	1362	1488
	36	0.932	11.22	1267	1360
	45	0.969	11.66	1232	1272
Betula lenta.....	12	1.411	16.97	1160	822
	29	1.518	18.25	1056	696
Betula lutea.....	11	1.050	12.63	990	942
	25	1.173	14.11	1083	923
	39	1.257	15.12	1110	883
	52	1.331	16.01	1027	772
	66	1.239	15.55	1160	897

extensive studies. We know that potassium in some way favors carbohydrate formation, and it may well be that ions retarding photosynthesis may be screened out in their passage through the conducting system, so that a more optimal mixture of inorganic ions is present at the higher levels.

Electrical conductivity has been used very extensively in measuring

changes in permeability. Osterhout[32-38] has done much of the preliminary work in this field. He has prepared a general summary[39] of a part of his work. Figure 98 shows the curves for electrical resistance of

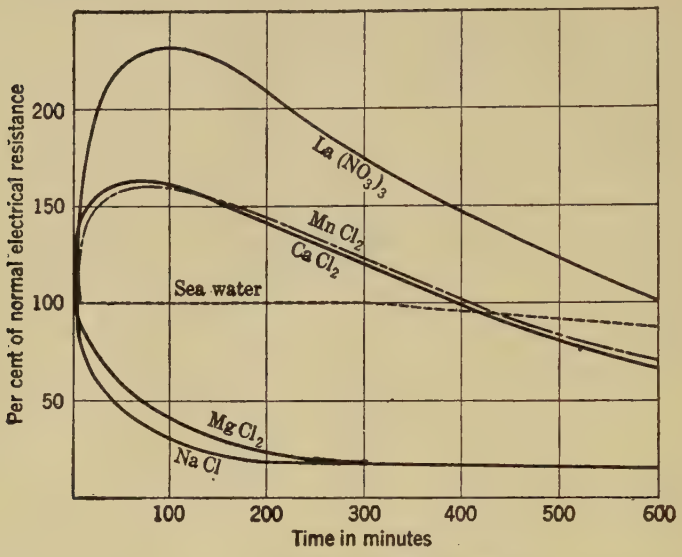


FIG. 98.—Showing the change in relative electrical resistance of *Laminaria agardhii* with time, in solutions having the same electrical conductivity as sea water. (Data of Osterhout.)

the sea algae, *Laminaria agardhii*, in electrolyte solutions having the same conductivity as sea water. It will be noted that the electrolytic resistance falls sharply in pure solutions of sodium chloride or magnesium

[32] Osterhout, W. J. V., Conductivity as a Measure of Permeability, *J. Biol. Chem.*, 36 : 485–487 (1918).

[33] Osterhout, W. J. V., A Method of Measuring the Electrical Conductivity of Living Tissues, *J. Biol. Chem.*, 36 : 557–568 (1918).

[34] Osterhout, W. J. V., Note on the Effect of Diffusion upon the Conductivity of Living Tissue, *J. Biol. Chem.*, 36 : 489–490 (1918).

[35] Osterhout, W. J. V., Some Quantitative Researches on the Permeability of Plant Cells, *Plant World*, 16 : 129–144 (1913).

[36] Osterhout, W. J. V., The Decrease in Permeability Produced by Anaesthetics, *Bot. Gaz.*, 61 : 148–158 (1916).

[37] Osterhout, W. J. V., A Comparative Study of Permeability in Plants, *J. Gen. Physiol.*, 1 : 299–304 (1919).

[38] Osterhout, W. J. V., A Comparison of Permeability in Plant and Animal Cells, *J. Gen. Physiol.*, 1 : 409–413 (1919).

[39] Osterhout, W. J. V., Injury, Recovery, and Death, in Relation to Conductivity and Permeability, J. B. Lippincott Company, Philadelphia (1922).

chloride, whereas it rises sharply in solutions of calcium chloride, manganous chloride, and lanthanum nitrate. In the last three solutions the initial rise is followed by a subsequent fall in resistance.

Osterhout notes that all of these curves are reversible in the initial part of the curve. Thus, for example, the resistance may be appreciably decreased by immersing the cells in sodium chloride solution, and recovery will take place if the cells are transferred to sea water. If, however, the decrease of conductivity is too great, recovery is no longer possible. Similarly, the resistance may be allowed to increase in calcium chloride solutions, and recovery to normal will take place. There is, however, a critical point past which recovery is no longer possible. Osterhout regards the curves in either direction as indicative of a death process and believes that death changes may be studied quantitatively by changes in electrical conductivity. It is thus possible to injure an organism, so that it behaves as though it were 5 per cent dead, 10 per cent dead, 20 per cent dead, etc., and its biochemical reactions under such conditions can be studied.

Similar studies have been made with animal tissues, for example, frog skin, and similar effects have been observed.

Clowes[40] interprets these results as changes in protoplasmic emulsions, due to the effect of the specific ions, regarding the plasma membrane as an emulsion fairly closely balanced between oil-in-water and water-in-oil types, the sodium chloride causing a more complete peptization of the cell membrane and forming a more perfect oil-in-water type, the calcium chloride causing inversion to a water-in-oil type. If water is the continuous phase, a low electrical resistance should result. If oil is the continuous phase, there would be a high electrical resistance. Eventually either calcium chloride or sodium chloride breaks the emulsion, causing a separation of the two phases with the ensuing death of the organism. This theory appears to have experimental backing, inasmuch as emulsions soaked up in filter paper show, to a considerable degree, conductivity phenomena similar to those shown in Fig. 98. However, the form of the curves likewise suggests electrokinetic changes, and it may well be that the proteins of the cell wall and protoplasm are involved, as well as the fats and lipides.

Recent studies of the effect of electric currents of varying frequency on electrical conductivity of biological tissues have thrown some doubt on certain of the earlier studies of electrical conductivity in biological systems. McClendon[41-43] has pointed out that very different values

[40] Clowes, G. H. A., Protoplasmic Equilibrium. I. Action of Antagonistic Electrolytes on Emulsions and Living Cells, *J. Phys. Chem.*, 20 : 407–451 (1916).

[41] McClendon, J. F., Colloidal Properties of the Surface of the Living Cell, Colloid

for electrical conductivity may be obtained when the frequency of the electric current is varied. However, Blinks[44] has studied the electrical conductivity of *Laminaria* using the same apparatus as used by Osterhout, and concludes, "We may therefore hold to the interpretation advanced by Osterhout that the observed resistance change is really a change in the permeability of protoplasm to ions. Since the resistance may rise 60 per cent or more above the normal value in sea-water, it is evident that there must be considerable ionic exchange in the normal state."

Crile and his coworkers[45-47] have used electrical conductivity to measure changes taking place in animal tissues, and from the experiments Crile has developed an electrochemical theory of living processes.

It would take us too far afield to adequately discuss the data and theories which he presents. In many respects the experimental data with animal tissues are more or less analogous to the findings of Osterhout and others, using plant tissues. Crile finds that many substances alter the electrical conductivity of animal tissues and that, in general, exhaustion from any cause, such as surgical shock, insomnia, emotion (fright), infection, etc., is characterized by a diminished conductivity of the brain and nervous tissue, activation, on the other hand, being characterized by an increased conductivity of the brain and nervous tissue, tending later to decrease as exhaustion approaches. The action of drugs, similarly, has definite effects on the electrical conductivity of the various tissues of the body.

Surface Conductance.—As already noted in the discussion of electrokinetic phenomena, Briggs found that the electrical conductivity of

Symposium Monograph, Vol. IV, pp. 224–233, Chemical Catalog Company, Inc., New York (1926).

[42] McClendon, J. F., Colloidal Properties of the Surface of the Living Cell, I. Conductivity of Blood to Direct Electric Currents, *J. Biol. Chem.*, 68 : 653–663 (1926).

[43] McClendon, J. F., Colloidal Properties of the Surface of the Living Cell, II. Electric Conductivity and Capacity of Blood to Alternating Currents of Long Duration and Varying in Frequency from 260 to 2,000,000 Cycles per Second, *J. Biol. Chem.*, 69 : 733–754 (1926). Cf. Remington, R. E., The High Frequency Wheatstone Bridge as a Tool in Cytological Studies; with Some Observations on the Resistance and Capacity of the Cells of the Beet Root. *Protoplasma*, 5 : 338–399 (1928).

[44] Blinks, L. R., High and Low Frequency Measurements with *Laminaria*, *Science*, 68 : 235 (1928).

[45] Crile, G. W., A Bipolar Theory of Living Processes, The Macmillan Company, New York (1926).

[46] Crile, G. W., An Electro-chemical Theory of Normal and Certain Pathological Processes, *Proc. Am. Phil. Soc.*, 60 : 546–552 (1921).

[47] Crile, G. W., Hosmer, H. R., and Rowland, A. F., The Electrical Conductivity of Animal Tissues under Normal and Pathological Conditions, *Am. J. Physiol.*, 60 : 59–106 (1922).

a colloid gel was not necessarily related to the presence or the concentration of ionized electrolytes. He found that a membrane of pure cellulose immersed in conductivity water acted as a fairly efficient conductor of an electric current, and was able to demonstrate that the conductance was not due to inorganic constituents present in the system. Thus, if a mixture of cellulose fibers in conductivity water was placed in a conductivity cell, and the fibers were allowed to settle below the level of the electrodes, a specific conductivity as low as 4×10^{-6} mhos was realized. On shaking the cell so as to bring the cellulose fibers into suspension between the electrodes, a very marked increase in specific conductivity could be obtained, and when the fibers again were allowed to settle, the water between the electrodes regained its original conductivity. The cell fibers apparently acted in the same manner as minute metallic particles would act in affording a conducting path for the electric current.

In a later paper, Briggs[48] has discussed the theory of surface conductance, pointing out that the considerations of Smoluchowski[49] are incorrect, in that he considered surface conductance to be a function of the ζ-potential. Briggs finds that surface conductance is independent of the ζ-potential, as illustrated in Fig. 99, where the solid lines represent ζ-potential curves, the dotted lines, surface conductance curves on the systems. Figure 100, likewise taken from the data of Briggs, shows that there is a definite lyotropic series of ions which influence surface conductance.

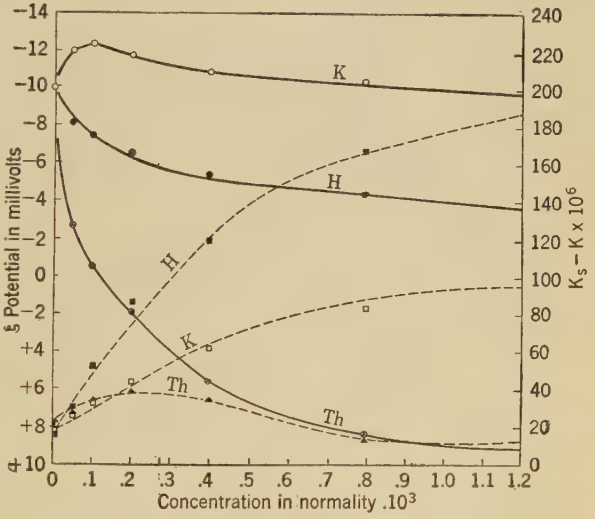


FIG. 99.—Showing that surface conductance is not a function of the electrokinetic potential for a cellulose membrane against salt solutions of varying concentrations. (Data of Briggs.)

[48] Briggs, D. R., Surface Conductance, Colloid Symposium Monograph, Vol. VI, pp. 41–52, Chemical Catalog Company, Inc., New York (1928).

[49] Smoluchowski, M. von, Zur Theorie der elektrischen Kataphorese und der Oberflächenleitung, *Physik. Z.*, 6 : 529–531 (1905).

In a study of various membranes, using the streaming potential cell shown in Fig. 51, Briggs found that certain systems, such as silica gel, showed high surface conductance, whereas other systems, such as flowers of sulfur, Al_2O_3, showed relatively slight surface conductance.

The theory of surface conductance still remains to be satisfactorily worked out. It appears that it may possibly be related to the specific surface area, *i.e.*, the colloidality of the material. Probably it is likewise influenced by the arrangement of the molecules of the dispersions medium or of materials present in true solution as oriented on the surface of the

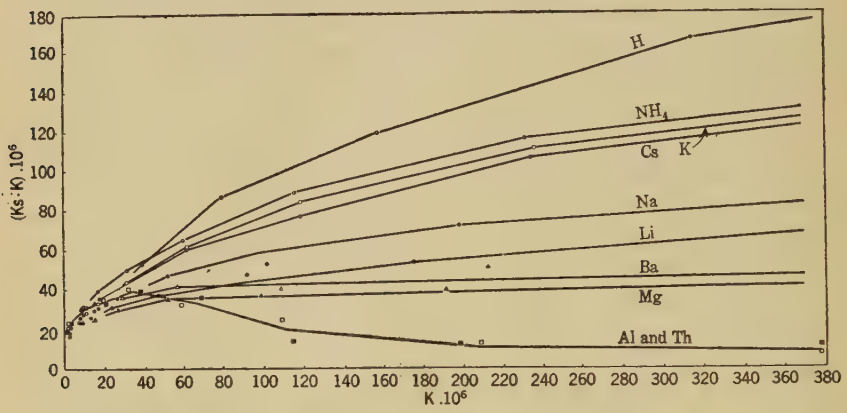


FIG. 100.—Illustrating the valence and lyotropic effect of cations upon the surface conductance of a cellulose membrane. (Data of Briggs.)

disperse phase. Briggs notes that surface conductance does not appear to be ionic conductance but instead is more nearly like metallic conductance.

In addition to specific surface area and molecular orientation, the affinity of a surface (wettability) for the dispersions medium will probably be found to play a role, and there is every indication that the specific conductivity of biological materials is in part due to surface conductance through the lyophilic colloid gel structure, as well as to ionic conductance through the liquid in the cells and intracellular spaces.

CHAPTER X

THE DONNAN EQUILIBRIUM

As already noted under the discussion of diffusion, two gases separated by a membrane permeable to both will diffuse through the membrane in both directions, so that at equilibrium the mixture will have the same composition on both sides of the membrane. Similarly, if a membrane separates two solutions of different concentration and if the membrane is permeable to both the solute and the solvent, equilibrium will be attained only when the concentration of the liquid on both sides of the membrane is identical. Thus, if a solution of potassium chloride is placed on one side of a permeable collodion membrane and a solution of equivalent concentration of sodium sulfate is placed on the opposite side of the membrane, at equilibrium it would be found that there is an equal concentration of potassium chloride and sodium sulfate on both sides of the membrane, assuming that the membrane is chemically inactive, merely acting as a septum to keep the initial solutions from mixing mechanically. Under such conditions, the osmotic pressure of the solutions in both compartments will be identical.

Donnan[1] notes, however, that very different conditions prevail when the membrane is impermeable to one of the ions. Assuming a compound, NaR, where R represents a colloid micelle, or an ion so large that it is unable to pass through the pores of the diaphragm, and assuming complete dissociation of the NaR into Na^+ and R^-, and further assuming complete dissociation of sodium chloride, and assuming equal volume of liquids on both sides of the membrane, we can represent the hypothetical initial state as:

Na^+	Na^+
R^-	Cl^-
(1)	(2)

[1] Donnan, F. G., Theorie der Membrangleichgewichte und Membranpotentiale bei Vorhandensein von nicht dialysierenden Elektrolyten. Ein Beitrag zur physikalisch-chemischen Physiologie, *Z. für Elektrochemie*, 17 : 572–581 (1911).

which at equilibrium becomes

Na^+	Na^+
R^-	Cl^-
Cl^-	
(1)	(2)

due to the diffusion of NaCl from compartment (2) through the membrane to compartment (1), the NaR being restrained from diffusing, due to the impermeability of the membrane to the anion.

In this equilibrium the necessary work for the iso-thermal reversible transference of a molecule of Na^+ from (2) to (1) is just as great as the work which is gained for the corresponding iso-thermal reversible transference of a molecule of Cl^-. Thus, if we consider the infinitely small iso-thermal and reversible change of the system in which *dn* molecules of Na^+ and *dn* molecules of Cl^- are transferred from (2) to (1), the work gained through this transference (the increase in free energy) is *nil*, and we can therefore write

$$dn \cdot RT \log_e \frac{(Na^+)_2}{(Na^+)_1} = - dn \cdot RT \log_e \frac{(Cl^-)_2}{(Cl^-)_1} \tag{94}$$

Dividing through by $(dn \cdot RT)$, we have the expression,

$$\log_e \frac{(Na^+)_2}{(Na^+)_1} = - \log_e \frac{(Cl^-)_2}{(Cl^-)_1} \tag{95}$$

or

$$\log_e (Na^+)_2 - \log_e (Na^+)_1 = - \log_e (Cl^-)_2 + \log_e (Cl^-)_1 \tag{96}$$

or

$$\log_e (Na^+)_2 + \log_e (Cl^-)_2 = \log_e (Cl^-)_1 + \log_e (Na^+)_1 \tag{97}$$

or

$$(Na^+)_2 \cdot (Cl^-)_2 = (Cl^-)_1 \cdot (Na^+)_1 \tag{98}$$

where the quantity within the parentheses means the molar concentration of the respective ions.

Equation (98) states that *the product of the sodium and chlorine ion concentrations in compartment* (2) *at equilibrium is equal to the product of the sodium and chlorine ion concentrations in compartment* (1). Since compartment (2) contains only sodium chloride at equilibrium, the chlorine ion concentration in compartment (2) must be equal to the sodium ion concentration.

Accordingly,

$$[(Cl^-)_2]^2 = (Na^+)_1 \cdot (Cl^-)_1 \tag{99}$$

Since at equilibrium NaR, as well as NaCl, is present in compart-

ment (1), the concentration of $(Na^+)_1$ will be greater than the concentration of $(Cl^-)_1$. This means that there will be an unequal distribution of ions on the two sides of the membrane at equilibrium, and accordingly *at equilibrium* the liquid on one side of the membrane may have an appreciably higher osmotic pressure than the liquid on the opposite side of the membrane.

Assuming that x molecules of sodium chloride diffused from compartment (2) to compartment (1), we would have the following concentrations of ions on the two sides of the membrane:

Original State				State of Equilibrium			
Na^+	R^-	Na^+	Cl^-	Na^+	$R^- \; Cl^-$	Na^+	Cl^-
C_1	C_1	C_2	C_2	$C_1 + x$	$C_1 \; x$	$C_2 - x$	$C_2 - x$
(1)		(2)		(1)		(2)	

where C_1 and C_2 are the original molar ion concentrations in compartments (1) and (2).

Equation (98) affords in this case, the following algebraic solution for the quantity of sodium chloride which is transferred from compartment (2) to compartment (1).

$$(C_1 + x) \cdot x = (C_2 - x)^2 \tag{100}$$

or

$$x = \frac{(C_2)^2}{C_1 + 2C_2} \tag{101}$$

and

$$\frac{x}{C_2} = \frac{C_2}{C_1 + 2C_2} \tag{102}$$

or

$$\frac{C_2 - x}{x} = \frac{C_1 + C_2}{C_2} \tag{103}$$

The per cent of sodium chloride which will diffuse from (2) to (1) is accordingly $\frac{x}{C_2} \cdot 100$, and the ratio of the division of sodium chloride between compartment (2) and compartment (1) at equilibrium is given by $\frac{C_2 - x}{x}$.

Tables XXXIX and XL show the distribution of sodium chloride at equilibrium under the conditions that we have been discussing, for various initial concentrations of NaR and sodium chloride in compartment (1) and compartment (2). These tables show that the action of non-dialyzable but electrolytically dissociated NaR is very important.

Although the membrane is completely permeable to sodium chloride, a high enough concentration of NaR will practically inhibit the diffusion of sodium chloride through the membrane. Conversely, if sodium chloride

TABLE XXXIX

The Distribution of Sodium Chloride on the Two Sides of a Membrane Permeable to Sodium Chloride but Impermeable to a Colloidal Ion (R^-) in the Solution on One Side of the Membrane (Calculations of Donnan).

Original State				State of Equilibrium				
Na^+ C_1	R^- C_1	Na^+ C_2	Cl^- C_2	Na^+ $C_1 + x$	R^- C_1	Cl^- x	Na^+ $C_2 - x$	Cl^- $C_2 - x$
1000	1000	1000	1000	1333	1000	333	666	666
1000	1000	100	100	1008	1000	8.3	92	92
100	100	1000	1000	576	100	476	524	524

TABLE XL

The Distribution Ratio of Sodium Chloride on the Two Sides of a Membrane Permeable to Sodium Chloride but Impermeable to a Colloidal Ion (R^-) in the Solution on One Side of the Membrane (Calculations of Donnan).

Original Concentration of NaR in (1) C_1	Original Concentration of NaCl in (2) C_2	Original Ratio of NaR to NaCl $\frac{C_1}{C_2}$	Per Cent NaCl Going from (2) to (1) $\frac{100\,x}{C_2}$	Ratio of NaCl between (2) and (1) $\frac{C_2 - x}{x}$
0.01	1.00	0.01	49.7	1.01
0.10	1.00	0.10	47.6	1.10
1.00	1.00	1.00	33.0	2.00
1.00	0.10	10.00	8.3	11.00
1.00	0.01	100.00	1.0	99.00 (?)

were added to the compartment containing the non-diffusible ion, the presence of the non-diffusible ion would cause the *excretion* of sodium chloride from this compartment, *such excretion taking place even against an opposing osmotic pressure.* The membrane which is freely permeable to sodium chloride thus shows an apparent differential permeability for the completely dialyzable sodium chloride, this differential permeability being due not to the membrane but to the ionic micelles in the sol on one side.

As Donnan points out, this phenomenon must play a role in physiology. Non-dialyzable anions and cations are present in the cell contents and in the intracellular fluids. The membranes may themselves be completely permeable to molecularly dissolved solutes, but due to the influence of the ionic micelles, an unequal distribution of ions occurs on the two sides of the membrane.

Donnan further takes up a consideration of the case of an electrolyte having no common ion with the ionic micelle. Considering, in this instance, NaR on one side of the membrane and potassium chloride on the opposite side of the membrane, we have the following, as the original and final states:

Original State		State of Equilibrium	
Na^+	K^+	Na^+	Na^+
R^-	Cl^-	K^+	K^+
		Cl^-	Cl^-
		R^-	
(1)	(2)	(1)	(2)

Here we have three kinds of iso-thermal reversible changes in the system:

(*a*) the transference of *dn* mol Na^+ from (1) to (2), and the transference of *dn* mol K^+ from (2) to (1).

Accordingly,

$$dn \cdot RT \log_e \frac{(Na^+)_1}{(Na^+)_2} + dn \cdot RT \log_e \frac{(K^+)_2}{(K^+)_1} = 0 \qquad (104)$$

from which by the same algebraic procedure that was used earlier,

$$\frac{(Na^+)_1}{(Na^+)_2} = \frac{(K^+)_1}{(K^+)_2} \qquad (105)$$

(*b*) the transference of *dn* mol Na^+ from (1) to (2), and the transference of *dn* mol Cl^- from (1) to (2).

Accordingly,

$$\frac{(Na^+)_1}{(Na^+)_2} = \frac{(Cl^-)_2}{(Cl^-)_1} \qquad (106)$$

(*c*) the transference of *dn* mol K^+ from (2) to (1), and the transference of *dn* mol Cl^- from (2) to (1).

Accordingly,

$$\frac{(K^+)_1}{(K^+)_2} = \frac{(Cl^-)_2}{(Cl^-)_1} \qquad (107)$$

By the use of simultaneous equations we can solve for the quantity of sodium chloride which appears in compartment (2) and for the quantity of potassium chloride which is transferred from compartment (2) to compartment (1), assuming again complete dissociation of the salts and equal volumes of liquid on the two sides of the membrane. The diffusion changes may be represented as follows:

Original State				State of Equilibrium						
Na^+	R^-	K^+	Cl^-	Na^+	K^+	Cl^-	R^-	K^+	Na^+	Cl^-
C_1	C_1	C_2	C_2	$C_1 - z$	x	y	C_1	$C_2 - x$	z	$C_2 - y$
(1)		(2)		(1)				(2)		

where z = the number of Na^+ ions transferred from (1) to (2);
x = the number of ions of K^+ transferred from (2) to (1);
y = the number of ions of Cl^- transferred from (2) to (1).

From equations (105), (106), and (107),

$$\frac{(Na^+)_1}{(Na^+)_2} = \frac{(K^+)_1}{(K^+)_2} = \frac{(Cl^-)_2}{(Cl^-)_1} \tag{108}$$

and by algebraic procedure Donnan finds the following values for (x), (y), and (z).

$$x = \frac{(C_1 + C_2)C_2}{C_1 + 2C_2} \tag{109}$$

$$y = \frac{(C_2)^2}{C_1 + 2C_2} \tag{110}$$

$$z = x - y \tag{111}$$

The above relationships allow us to solve for the actual distribution at equilibrium, as shown in Table XLI. Referring to this table, it will be noted that there are very great differences in the composition of the equilibrium solution on the two sides of the membrane and that these differences depend very largely upon the initial concentrations. This is more clearly shown in Table XLII.

It will be noted that when a relatively large amount of NaR is on one side of the membrane with a relatively small amount of potassium chloride on the other side of the membrane, there is almost complete transference of the K^+ ions from (2) to (1). Thus, a high concentration of NaR in (1) will separate almost completely the K^+ from the Cl^- with

TABLE XLI

Showing the Original Concentrations and Equilibrium Concentrations of a System of NaR and KCl Separated by a Membrane Impermeable to the Anion (R^-) but Freely Permeable to the Other Ions

Original State				State of Equilibrium						
Na^+ C_1	R^- C_1	K^+ C_2	Cl^- C_2	Na^+ $C_1 - z$	K^+ x	R^- C_1	Cl^- y	K^+ $C_2 - x$	Na^+ z	Cl^- $C_2 - y$
1000	1000	1000	1000	666	666	1000	333	333	333	666
1000	1000	100	100	916.7	91.7	1000	8.3	8.3	83.4	91.7
100	100	1000	1000	52	524	100	476	476	48	524

TABLE XLII

Showing the Change in the Equilibrium Concentration of Ions on the Two Sides of a Membrane When the Membrane is Impermeable to One of the Ions Originally Present in Compartment (C_1).

Ratio of Original Concentration, $C_1 : C_2$	Ratio of Equilibrium Concentration, $C_1 : C_2$
1 : 1	2 : 1
10 : 1	11 : 1
1 : 10	1.1 : 1

which it was originally associated, carrying the K^+ across the membrane and selectively leaving the Cl^- behind. *We have here an excretion of sodium and the selective adsorption of potassium across a membrane, due not to a differential permeability of the membrane but rather to the presence of ionic micelles on one side of the membrane.* It is entirely probable that the selective adsorption of potassium by plants from the soil solution or by marine algae from the sea water is due to a Donnan equilibrium, such as is noted in the above calculations. It may well be that some similar mechanism accounts for the excretion of urea through the tubules of the kidneys which appear to have a one-sided permeability.

Donnan continues his discussion, taking up the question of the hydrolytic decomposition of salts through the action of a membrane. If NaR is placed on one side of a membrane and pure water is placed on the opposite side, we should have, due to the presence of hydrogen and hydroxyl ions in the water, the following original and equilibrium conditions.

Original State		State of Equilibrium	
Na^+	H^+	Na^+	Na^+
R^-	OH^-	H^+	OH^-
(1)	(2)	R^-	
		(1)	(2)

Here Donnan shows that

$$\frac{(Na^+)_1}{(Na^+)_2} = \frac{(OH^-)_2}{(OH^-)_1} \tag{112}$$

Assuming the original and equilibrium states of

Original State			State of Equilibrium				
Na^+	R^-		Na^+	H^+	R^-	Na^+	OH^-
C_1	C_1	Pure Water	$C_1 - x$	x	C_1	x	x
(1)		(2)	(1)			(2)	

the value of (x) can be determined from the dissociation constant of water.

$$x \cdot (OH^-)_1 = K_w \tag{113}$$

from which Donnan obtains the value of (x) as

$$x = \sqrt[3]{K_w C_1} \tag{114}$$

Table XLIII shows Donnan's calculations for the value of (x) at various concentrations of NaR and the percentage of Na^+ which is transferred across the membrane to form NaOH.

TABLE XLIII

Showing the Hydroxyl Ion Concentration (x) Outside of a Membrane at Equilibrium and the Percentage of $Na^+ \left(\frac{100\,x}{C_1}\right)$ Transferred Across the Membrane for Various Original Concentrations of NaR on One Side of the Membrane and of Pure Water on the Other Side of the Membrane.

C_1	x	$\frac{100\,x}{C_1}$
		Per Cent
0.01	5.10^{-6}	0.05
0.10	1.10^{-5}	0.01
1.00	2.10^{-5}	0.002

In this case, one would have alkali excreted through the membrane, the liquid on the inside of the membrane becoming more acid. On the

other hand, assuming a positively charged ionic micelle, we might very well have the following initial state and equilibrium state:

Original State		State of Equilibrium	
R^+	H^+	R^+	H^+
Cl^-	OH^-	Cl^-	Cl^-
(1)	(2)	OH^-	
		(1)	(2)

In this case we should have *acid excreted* across the membrane.

Donnan points out that *the proper ampholyte can easily give rise by this means alone to a concentration of hydrogen ions in the external liquid as great as that found in the gastric juice.*

Donnan[2-4] has subjected this theory to experimental studies and finds that the theoretical considerations are realized experimentally.

Loeb[5] made a series of elaborate studies of the influence of acids, bases, and salts on protein systems, and because of the fact that such systems obeyed the Donnan equilibrium, he came to the conclusion that acids and bases, and in some instances salts, combined in stoichiometrical proportion with the proteins, forming definite compounds which could be characterized as "protein chloride," "sodium proteinate," etc. It may eventually be demonstrated that Loeb was correct in assuming a stoichiometrical combination between the protein and the acid or the base. The fact that a definite Donnan equilibrium is set up, however, is no proof of such a relationship, as has already been indicated by Hill,[6] and by Rinde.[7]

While the NaR or the RCl of the hypothetical compounds which are placed on one side of the membrane may represent chemical individuals, non-diffusible anions or cations, respectively, it is equally possible that an adsorption complex, such as we have already postulated in a con-

[2] Donnan, F. G., and Garner, W. E., Equilibria across a Copper Ferrocyanide and an Amyl Alcohol Membrane, *J. Chem. Soc.*, London, 115 : 1313–1328 (1919).

[3] Donnan, F. G., and Allmand, A. J., Ionic Equilibria across Semipermeable Membranes, *J. Chem. Soc.*, London, 105 : 1941–1963 (1914).

[4] Donnan, F. G., and Harris, A. B., The Osmotic Pressure and Conductivity of Aqueous Solutions of Congo Red, and Reversible Membrane Equilibria, *J. Chem. Soc.*, London, 99 : 1554–1577 (1911).

[5] Loeb, Jacques, Proteins and the Theory of Colloidal Behavior, McGraw-Hill Book Company, New York (1922). (Cf. also numerous articles in the *Journal of General Physiology*, Vols. 1–6, inclusive.)

[6] Hill, A. V., The Potential Difference Occurring in a Donnan Equilibrium and the Theory of Colloidal Behavior, *Proc. Roy. Soc.*, (A)102: 705–710 (1923).

[7] Rinde, H., A Method for the Determination of the Adsorption of Ions on Colloidal Particles by Means of Donnan's Membrane Equilibrium Theory, *Phil. Mag.*, (7) 1 : 32–50 (1926).

sideration of the complex theory of colloids, could give rise to a non-diffusible ion and a diffusible ion. Thus, a ferric hydroxide sol, stabilized by *adsorbed* ferric chloride, would yield an ionic micelle which could be represented by $([Fe(OH)_3]_x\ [FeCl_3]_y)^+$ and Cl^-, or the ionic micelle might acquire a positive charge, due to the adsorption of a ferric ion. Its composition then would be represented by $[Fe(OH)_3]_x\ Fe^{+++}$. In either instance we would have an ionic micelle for which no stoichiometrical formula could be written. If, however, such a micelle were on one side of the membrane, it would behave as a non-diffusible ion and produce a Donnan equilibrium. Accordingly *a Donnan equilibrium is set up whenever colloidal micelles, carrying either a positive or a negative charge, are concentrated on one side of a membrane, the membrane being impermeable to such micelles but permeable to the ions of an electrolyte on the other side of the membrane.*

An inspection of the preceding tables, showing the unequal distribution of diffusible ions on the two sides of the membrane, probably accounts in a considerable measure for the concentration of electrolytes within plant or animal cells and tissues. Contrary to what one would expect, *the Donnan equilibrium provides a mechanism whereby such cells and tissues may possess a considerably higher osmotic pressure than the liquid which bathes them and at the same time be in equilibrium with the external liquid.*

However, one must not consider that the Donnan equilibrium offers a complete explanation for the adsorption of ions from the soil solution by the root hairs of plants. While probably it plays a role, there are other, as yet unknown, factors involved. *The cell membrane, at least in certain instances, is more than a mechanical sieve restraining the diffusion of ionic micelles.* Harris and his coworkers[8-12] have shown that the leaf tissue fluids of Egyptian and Upland cotton in the Gila river valley of Arizona differ very markedly in the content of chloride and sulfate ions,

[8] Harris, J. A., Lawrence, J. V., and Lawrence, Z. W., The Chlorid Content of the Leaf Tissue Fluids of Egyptian and Upland Cotton, *J. Agr. Res.*, 28 : 695–704 (1924).

[9] Harris, J. A., Hoffman, C. T., and Hoffman, W. F., Sulphate Content of the Leaf-Tissue Fluids of Egyptian and Upland Cotton, *J. Agr. Res.*, 31 : 653–661 (1925).

[10] Harris, J. A., Hoffman, W. F., Sinclair, W. B., Johnson, A. H., and Evans, R. D., The Leaf-Tissue Fluids of Egyptian Cottons, *J. Agr. Res.*, 31 : 1027–1033 (1925).

[11] Harris, J. A., Hoffman, W. F., and Lawrence, J. V., Differential Absorption of Anions by Varieties of Cotton, *Proc. Soc. Exp. Biol. Med.*, 22 : 350–352 (1925).

[12] Harris, J. A., The Accumulation of Chlorides in the Leaf Tissue Fluids of Egyptian Cotton with the March of the Season, *Proc. Soc. Exp. Biol. Med.*, 22 : 415–417 (1925).

TABLE XLIV

Comparison of Chloride and Sulfate Content of Leaf Tissue Fluids of Egyptian ("Pima") and Upland ("Lone Star") Cottons as Grown at Sacaton, Arizona, during 1923 (Data of Harris, Hoffman, and Lawrence).

	N	Mean Content for Egyptian Cotton	Mean Content for Upland Cotton	Difference between Egyptian and Upland Cotton		
				Absolute Difference and Probable Error	Ratio of Absolute Difference to Probable Error	Percentage Difference
First series of determinations: July 29 to August 14.						
Grams Chloride per Liter.....	68	2.4412 ± .0406	1.0515 ± .0239	+1.3897 ± .0396	35.1	+79.6
Grams Sulfate per Liter.....	67	12.5373 ± .1022	16.2164 ± .0801	−3.6791 ± .1410	26.1	−25.6
Second series of determinations: August 18 to August 31.						
Grams Chloride per Liter.....	68	3.3088 ± .0477	1.1434 ± .0302	+2.1654 ± .0520	41.7	+97.3
Grams Sulfate per Liter.....	70	14.1642 ± .1294	17.3357 ± .1793	−3.1714 ± .1750	18.2	−20.1

the Egyptian type having a chloride content from 28 to 189 per cent higher than the Upland cottons, whereas the Upland cottons have a sulfate content 18 to 28 per cent higher than the Egyptian cottons. Table XLIV shows certain of their data.

The surprising feature of these investigations is that *the ability of the plant to selectively absorb sulfates and chlorides from the saline soil solution is heritable.* The F_1 crosses between Egyptian and Upland types yielded progeny which were intermediate between the parents in this respect, whereas the ability to selectively absorb either chlorides or sulfates segregated in the F_2 generation.

In the experiments of Harris and his coworkers there was no possibility that the results were due to soil heterogeneity. The plants under experiment were alternated in the row and almost invariably showed the higher sulfate or higher chloride content characteristic of the variety. Similarly, the F_1 generation and the F_2 generation were spaced methodically over the fields and still showed in the one case (F_1) intermediate characteristics and in the other (F_2), the characteristic segregation. The physical mechanism responsible for the selective accumulation of sulfates in the tissue fluids of one form and of a preponderance of chlorides in the tissue fluids of the other form still remains to be worked out.

II

PROTEINS

"Da die Proteïnstoffe bei allen chemischen Prozessen im lebenden Organismus auf die eine oder andere Weise beteiligt sind, so darf man von der Aufklärung ihrer Struktur und ihrer Metamorphosen die wichtigsten Aufschlüsse für die biologische Chemie erwarten."

EMIL FISCHER (1906)

CHAPTER XI

THE AMINO ACIDS, THE PRIMARY DECOMPOSITION PRODUCTS OF PROTEINS

Early Investigations. — With the remarkable development of organic chemistry which began early in the nineteenth century it is not surprising that the attention of the chemist was directed toward a study of the proteins, inasmuch as proteins are a major constituent of every living cell.

The methods which the organic chemist developed, *i.e.*, the elementary analyses for C, H, N, O, and S, were early applied, but it was impossible, due to the complex nature of the proteins, to differentiate the proteins on the basis of their elementary analysis. Accordingly the early chemists reverted to the other analytical procedures of the organic laboratory. Proteins were subjected to oxidation, using chromic acid, neutral solutions of potassium permanganate, alkali solutions of potassium permanganate; to reduction, using tin and hydrochloric acid, hydriodic acid, etc.; to destructive distillation; to fusion with alkalies; to the action of concentrated and fuming nitric acid in attempts to prepare nitro derivatives; to the action of halogens in attempts to prepare characteristic halogen derivatives; to sulfonation; in fact, to all of the usual procedures of the organic laboratory.

At the time these studies were carried out nothing was known of the chemical nature of the proteins, and accordingly it is not surprising that little information was gained from the application of such technic. Now that we have an insight into the chemical nature of the proteins, and accordingly have some idea as to what products might be formed by the actions of the various reagents, it would be highly desirable to repeat many of these older studies in the light of modern information and modern technic. To some extent this is being done at the present time.

Johnson[1] has successfully nitrated various proteins, and Johnson,[2]

[1] Johnson, T. B., et al., Studies on Nitrated Proteins, I–V, *J. Am. Chem. Soc.*, 37 : 1863–1884, 2164–2170, 2170–2178, 2598–2603 (1915); 38 : 1392–1398 (1916).

[2] Johnson, T. B., and Daschavsky, P. G., Researches on Proteins. VI. Destructive Distillation of Fibroin, *J. Am. Chem. Soc.*, 41 : 1147–1449 (1919).

and Pictet[3] have subjected proteins to destructive distillation, securing valuable data. Similarly, Vandevelde[4] is studying the halogen derivatives of the proteins.

In 1848, Guckelberger,[5] a pupil of Liebig, oxidized egg white, casein, fibrin, and gelatin with potassium permanganate, and potassium bichromate and sulfuric acid. Among the products obtained he identified formic, acetic, propionic, valeric, caproic, and benzoic acids, as well as benzaldehyde and ammonia. He was, however, unable to correlate these findings with any rational structure of the protein molecule.

Bernert,[6] using potassium permanganate in alkaline solution, obtained the same list and in addition, pyrrol and two amino acids, lysine and histidine. Kutscher[7] obtained guanidine-butyric acid and succinic acid. Lossen[8] had earlier obtained guanidine from proteins by oxidation.

Other workers, using hydrogen peroxide in acid solution with ferrous sulfate as a catalyst, obtained acetone and iso-valeric aldehyde.

Hlasiwetz and Habermann[9] treated egg albumin with free bromine in an autoclave, and from 100 grams of egg albumin obtained 29.9 grams of bromoform, 22 grams of bromacetic acid, 12 grams of oxalic acid, 23.8 grams of aspartic acid, 22.6 grams of leucine, and 1.5 grams of bromanil (tetra brom *p.* benzoquinone).

Mann[10] has discussed some of these earlier investigations at considerable length. Suffice it to say that while all of these studies and many other studies, similar to those which have been noted, showed the great complexity of the protein molecule. they did not contribute much

[3] Pictet, A., and Cramer, M., Sur la distillation de l'ovalbumine sous pression réduite, *Helv. Chim. Acta.*, 2 : 188–195 (1919).

[4] Vandevelde, A. J. J., Contribution a l'étude des protéines halogénées, I–V, *Rec. trav. chim.*, 43 : 158–162, 326–328, 702–706 (1924); 44 : 224–228, 900–906 (1925); Halogenated Proteins, VI–VIII, *Rec. trav. chim.*, 45 : 825–829 (1926); 46 : 133–136, 590–593 (1927).

[5] Guckelberger, G., Ueber einige flüchtige Zersetzungsproducte des Albumens, Fibrins, Caseins und des Leims durch Manganhyperoxyd und Chromsäure, unter Mitwirkung von Schwefelsäure, *Ann.*, 64 : 39–100 (1848).

[6] Bernert, R., Ueber Oxydation von Eiweiss mit Kaliumpermanganat, *Z. physiol. Chem.*, 26 : 272–307 (1898).

[7] Kutscher, Fr., Die Oxydationsprodukte des Arginins. II. *Z. physiol. Chem.*, 32 : 413–418 (1901).

[8] Lossen, F., Guanidin, ein Oxydationsproducte des Eiweisses, *Ann.*, 201 : 369–376 (1880).

[9] Hlasiwetz, H., and Habermann, J., Ueber die Proteinstoffe, *Ann.*, 159 : 304–333 (1871).

[10] Mann, Gustav, Chemistry of the Proteids, Macmillan and Company, London (1906).

in the way of establishing the essential chemical nature of the proteins or the structure of the protein molecule.

The Amino Acids as Decomposition Products of Proteins.—We now know the protein molecule to be made up primarily of amino acids linked together through the amino and carboxyl groups or through other reactive groups present in the amino acids concerned. The real clue to the structure of the proteins came from investigations where hydrolytic methods (adding the elements of water) were employed, hydrolyzing the protein with either (*a*) acids, or (*b*) enzymes.

Braconnot[11] was apparently the first investigator to use acid hydrolysis. He boiled both gelatin and meat with dilute sulfuric acid and identified glycine (glycocoll, $CH_2(NH_2)COOH$, amino acetic acid) from both proteins. This was the first instance in which an amino acid was shown to be a primary decomposition product of proteins.

Proust[12] had earlier isolated leucine (β-iso-propyl α-amino propionic acid) from cheese and had called it "oxide caséeux." While this was the first amino acid to be discovered, the fact that it was present in crystalline form in the holes in the cheese did not prove that it was a primary decomposition product of the protein.

Braconnot's method of acid hydrolysis was not generally recognized as affording a means by which primary protein decomposition products could be obtained, and no further amino acids were discovered until Liebig,[13] in 1846, isolated a crystalline compound from casein which had been hydrolyzed by strong alkali. Bopp,[14] and Hinterberger[15] later identified this compound as tyrosine, β-(*p.* hydroxy phenyl) α-amino propionic acid. The next amino acid to be discovered was serine (β-hydroxy α-amino propionic acid), isolated by Cramer,[16] by hydrolyzing silk with sulfuric acid.

Kuhne,[17] in 1867, introduced a new method for the study of proteins, *i.e.*, *digestion with tryptic enzymes*, and identified tyrosine and leucine as primary decomposition products of proteins. Here again,

[11] Braconnot, H., Sur la conversion des matières animales en nouvelles substances par le moyen de l'acide sulfurique, *Ann. Chim. et Phys.*, 13 : 113–125 (1820).

[12] Proust, M., Recherches sur le principe qui assaisonne les fromages, *Ann. Chim. et Phys.*, 10 : 29–49 (1818).

[13] Liebig, J., Baldriansäure und ein neuer Körper aus Käsestoff, *Ann.*, 57 : 127–129 (1846).

[14] Bopp, F., Einiges über Albumin, Casein und Fibrin, *Ann.*, 69 : 16–37 (1849).

[15] Hinterberger, F., Untersuchung des Ochsenhorns, *Ann.*, 71 : 70–79 (1849).

[16] Cramer, E., Ueber die Bestandtheile der Seide, *J. prakt. Chem.*, (1) 96 : 76–98 (1865).

[17] Kuhne, W., Ueber die Verdauung der Eiweissstoffe durch den Pankreassaft, *Arch. für path. Anat. u. Physiol.*, 39 : 130–174 (1867).

however, the fact that tryptic digestion afforded a new technic for the study of proteins was not generally recognized.

At about this time, Ritthausen[18-19] began his extensive series of investigations of the vegetable proteins, and, in 1868 added aspartic acid (α-amino succinic acid) and glutamic acid (α-amino glutaric acid) to the list of known amino acids. These acids were isolated from vegetable proteins by acid hydrolysis. Schützenberger and Bourgeois[20] isolated alanine (α-amino propionic acid) from silk by hydrolysis with barium hydroxide, and its presence was later confirmed by Weyl,[21] using acid hydrolysis. Schulze and Barbieri[22] isolated phenyl alanine (β-phenyl α-amino propionic acid) from the sap of germinated seeds of *Lupinus luteus*, and, in 1889, Drechsel[23] identified lysine (α-ϵ-di-amino caproic acid) as a constituent of proteins.

Thus, in the period from 1800 to 1890, only nine amino acids were isolated and identified. During the decade, 1890 to 1900, four additional amino acids were isolated. In the decade, 1900 to 1910, the discovery of eight amino acids was announced, five of which, tryptophane, proline, oxy-proline, iso-leucine, and valine, are of rather common occurrence. Of the other three, one (β-alanine) has been confirmed but has not as yet been shown to be present in unaltered proteins. Another, di-amino tri-oxy dodecanoic acid ($C_{12}H_{26}N_2O_5$), has been considered extremely doubtful, but the recent announcement of Fränkel and Friedmann[24] of the isolation of an amino acid having the formula, $C_{10}H_{18}$—$(NH_2)_2$—$(COOH)_2 + H_2O$, can probably be regarded as a confirmation of this compound. Fränkel and Friedmann suggest that this compound may be identical with Skraup's[25] caseanic acid ($C_{12}H_{24}O_5N_2$). The third

[18] Ritthausen, H., Ueber das Pflanzen-Casein oder Legumin. II. Die Proteinsubstanz der Erbsen, Wicken, Saubohnen, Linsen, und Bohnen, das Pflanzen-Casein oder Legumin, *J. prakt. Chem.*, 103 : 193–216 (1868).

[19] Ritthausen, H., Asparaginsäure und Glutaminsäure, Zersetzungsprodukte des Legumins und Conglutins beim Kochen mit Schwefelsäure, *J. prakt. Chem.*, 107 : 218–240 (1869).

[20] Schützenberger, P., and Bourgeois, A., Recherches sur la constitution de la fibroine et de la soie, *Compt. rend.*, 81 : 1191–1193 (1875).

[21] Weyl, Th., Zur Kenntniss der Seide. II. *Ber.*, 21 : 1529–1532 (1888).

[22] Schulze, E., and Barbieri, J., Ueber Phenylamidopropionsäure, Amidovaleriansäure und einige andere stickstoffhaltige Bestandtheile der Keimlinge von Lupinus luteus, *J. prakt. Chem.*, (2) 27 : 337–362 (1883).

[23] Drechsel, E., Zur Kenntnis der Spaltungsprodukte des Caseins, *J. prakt. Chem.*, (2) 39 : 425–429 (1889).

[24] Fränkel, S., and Friedmann, M., Über eine Dodecandiaminodicarbonsäure aus Casein, *Biochem. Z.*, 182 : 434–441 (1927).

[25] Skraup, Zd. H., Über die Hydrolyse des Caseins durch Salzsäure, *Z. physiol. Chem.*, 42 : 274–296 (1904).

TABLE XLV

A List of the Naturally-Occurring Amino Acids
(The existence of a few of these amino acids is doubtful, as noted by "?")

Year	Common Name and Scientific Name (When Known)	Discoverer
1818	Leucine, β-iso-propyl-α-amino-propionic acid	Proust
1820	Glycine or glycocoll, α-amino-acetic acid	Braconnot
1846	Tyrosine, β-(*p.*-hydroxyphenyl) α-amino-propionic acid	Liebig
1865	Serine, β-hydroxy-α-amino-propionic acid	Cramer
1868	Aspartic acid, α-amino-succinic acid	Ritthausen
1868	Glutamic acid, α-amino-glutaric acid	Ritthausen
1875	Alanine, α-amino-propionic acid	Schützenberger and Bourgeois
1883	Phenylalanine, β-phenyl-α-amino-propionic acid	Schulze
1889	Lysine, α-ϵ-di-amino-caproic acid	Drechsel
1895	Arginine, α-amino-δ-guanidine valerianic acid	Hedin
1896	Histidine, β-imidazole-α-amino-propionic acid	Kossel
1896	3.5-di-iodio tyrosine	Drechsel
1899	Cystine, β-β'-di-thio-di-(α-amino propionic acid)	Mörner
1901	Tryptophane, β-indol-α-amino-propionic acid	Hopkins and Cole
1901	Proline, pyrrolidine-α-carboxylic acid	Fischer
1901	Cysteine, α-amino-β-thio-lactic acid	Embden
1902	Hydroxy-proline, hydroxy-pyrrolidine-α-carboxylic acid	Fischer
* 1904	Caseanic acid, $C_{12}H_{24}N_2O_5$ (?)	Skraup
* 1904	Diamino-tri-hydroxy-dodecanoic acid, $C_{12}H_{26}N_2O_5$ (?)	Fischer and Abderhalden
* 1927	Dodecan-diamino-di-carboxylic acid, $C_{12}H_{24}N_2O_4 \cdot H_2O$	Fränkel and Friedmann
1905	Iso-leucine, β-methyl-β-ethyl-α-amino-propionic acid	Winterstein
1906	Valine, α-amino-iso-valerianic acid	Fischer
* 1907	Hydroxy-tryptophane [position of (—OH) uncertain (?)]	Abderhalden and Kempe
* 1924	β-Bz.-hydroxy-Pr.-dihydro-indolyl-alanine	Abderhalden and Sickel
1908	β-alanine, β-amino-propionic acid	Engeland
1913	Nor-leucine, α-amino *n.* caproic acid	Abderhalden and Weyl
1913	α-amino *n.* butyric acid	Foreman
1913	3.4-di-hydroxy-phenylalanine (dopa)	Guggenheim
1918	β-hydroxy-glutamic acid	Dakin
1919	Thyroxine, β-(3.5.3′5′ tetra-iodio 4′ hydroxy-di-phenyl-ether) α-amino-propionic acid	Kendall
1923	$C_5H_{11}SNO_2$	Mueller
1925	$C_4H_{11}O_3N$(?)	Gortner and Hoffman
1925	$C_6H_{14}N_2O_3$ (Probably hydroxy-lysine (?))	Schryver
1926	$C_4H_9NO_3$ (Possibly hydroxy-amino-butyric acid (?))	Schryver and Buston
1926	$C_5H_{11}NO_3$ (hydroxy-valine (?))	Schryver and Buston

* The compounds within the bracket are probably identical.

amino acid, oxytryptophane,[26] is probably[27] β-Bz-oxy-Pr-dihydroindolylalanine ($C_{12}H_{14}O_3N_2$).

Since 1910, nine additional amino acids have been announced. One of these (dihydroxyphenylalanine) was identified by Guggenheim[28] as occurring free in the pods of *Vicia faba*, and was again isolated by Miller[29] from the velvet bean, *Stizolobium deeringianum*. It has not been obtained as a primary decomposition product of proteins, although there is certain evidence that it may occur in proteins, but, due to its labile nature, be destroyed in the process of hydrolysis.

Table XLV lists the various amino acids, the year of their discovery, their common names, and their scientific names, with the names of the persons making the announcement of discovery.

Cysteine (α-amino β-thio lactic acid), isolated by Embden,[30] is included in the above list. It has been until recently regarded as a decomposition product of cystine. However, Kozlowski[31] has identified cysteine as occurring in the free state in the green hulls and the immature seeds of the common pea (*Pisum sativum*), but it has not been isolated from proteins. However, the usual methods of protein study would cause oxidation of cysteine to cystine which may account for the fact that it has not been generally recognized in the unaltered protein. This conclusion is verified by the observation of Okuda[32] who believes that both cysteine and cystine are primary decomposition products of proteins.

Figure 101 shows graphically the progress by decades of the isolation of new amino acids.

Including cysteine and all of the amino acids noted in Table XLV, the presence of 31 naturally occurring amino acids is indicated. There is no reason for believing that this completes the list of amino acids which

[26] Abderhalden, E., and Kempe, M., Beitrag zur Kenntnis des Tryptophans und einiger seiner Derivate, *Z. physiol. Chem.*, 52 : 207–218 (1907).

[27] Abderhalden, E., and Sickel, H., Isolierung einer Aminosäure der Indolreihe der Zusammensetzung $C_{11}H_{14}O_3N_2$ aus Casein, *Z. physiol. Chem.*, 138 : 108–117 (1924).

[28] Guggenheim, M., Dioxyphenylalanin, eine neue Aminosäure aus Vicia faba, *Z. physiol. Chem.*, 88 : 276–284 (1913).

[29] Miller, E. R., Dihydroxyphenylalanine, A Constituent of the Velvet Bean, *J. Biol. Chem.*, 44 : 481–486 (1920).

[30] Embden, G., Ueber den Nachweis von Cystin und Cystein unter den Spaltungsprodukten der Eiweisskörper, *Z. physiol. Chem.*, 32 : 94–103 (1901).

[31] Kozlowski, A., On the Non-Protein Cysteine in Plants. Preliminary Note on the Attempted Isolation of Glutathione from the Pea (*Pisum Sativum*), *Biochem. J.*, 20 : 1346–1350 (1926).

[32] Okuda, Y., On the Presence of Cysteine Group in Protein Molecules, *Proc. Imp. Acad.*, Japan. 2 : 277–279 (1926).

are present in the protein molecule. Probably no protein has been as extensively investigated as has casein, and the inadequacy of our present methods of protein research are strikingly illustrated by the fact that Dakin,[33] in 1918, isolated more than 10 per cent of β-hydroxy glutamic acid from casein. The amino acids which have been actually isolated from proteins in very few instances total more than 70 per cent of the

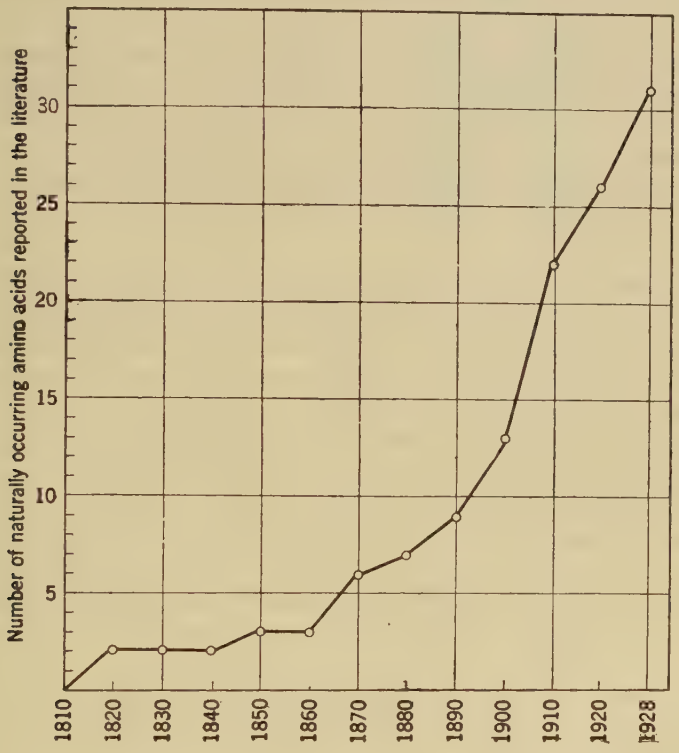


FIG. 101.—A graphic representation of the isolation of the naturally-occurring amino acids plotted against time.

original protein, and accordingly there is every reason for believing that there are a number of amino acids as yet unknown.

OUTSTANDING PROTEIN INVESTIGATORS.—Several names stand out prominently in protein research. The foremost of these is that of Emil Fischer who contributed so extensively to this field during the period from 1889 to 1918. The name of Emil Fischer is preeminent not alone in the field of proteins but likewise in the study of carbohydrates, the

[33] Dakin, H. D., On Amino-Acids, *Biochem. J.*, 12 : 290–317 (1918).

purine and pyrimidine derivatives, and of the tannins. Probably no one person has ever influenced the fields of biochemistry and organic chemistry to a greater extent than did Emil Fischer.

Fischer began his work on the proteins at a time when nine mono-amino acids and four di-amino acids were known. Eight of the mono-amino acids had previously been synthesized and their structures thus definitely proven. Serine and the di-amino acids had not been synthesized. It is an axiom of the organic chemist that until an organic compound has been synthesized by known chemical reactions, its structure is still uncertain. One of Fischer's tasks, accordingly, was to synthesize in the chemical laboratory those amino acids which had not been previously synthesized, in order to definitely prove the structures.

Fischer,[34] in 1906, presented an outline of the plan which he developed for the study of the proteins. The first item of the outline is these synthetic studies.

As a second field, he devoted himself to a study of amino acid derivatives, including the preparation and study of the esters and acetyl derivatives, and the phenylisocyanate derivatives, etc., with a view to finding chemical derivatives which would permit of the separation of mixtures of amino acids.

Having made a study of the amino acid derivatives, he then proceeded to the experimental separation of mixtures of amino acids, such as occur by the hydrolysis of unaltered proteins.

Simultaneously with his studies on the separation of the amino acids, he began investigations attempting to re-combine two or more amino acids into compounds which he designated by the name of " polypeptides " and studied the chemical and physical properties of such derivatives, particularly in their relation to protein structure. These polypeptides were subjected to hydrolysis, were tested as to their behavior toward enzyme action, and in various ways contributed much to our knowledge of the chemistry of the proteins.

A further field which Fischer extensively investigated was the study of the proteins themselves. He was interested in the problem of protein structure, in a critical study of the various methods for protein hydrolysis, conducted extensive series of analyses of various proteins, and studied in a general way the protein molecule as an entity. Remarkable as it may seem, he completed very satisfactorily and in a most extraordinary manner the entire program which he planned, and today we are indebted very largely to the technic of Emil Fischer for much of the available information on the organic chemistry of the protein molecule.

[34] Fischer, E., Untersuchungen über Aminosäuren, Polypeptide und Proteine, *Ber.*, 39 : 530–610 (1906).

Kossel, working during the period of 1895 to 1915, made a distinct contribution to protein chemistry in his study of the di-amino acids, including the Kossel method for their quantitative separation and identification (*vide infra*). He likewise contributed much to the general field of protein chemistry but was particularly interested in the histones and the protamines, the basic proteins characterized by a high content of di-amino acids.

Abderhalden received his basic training in the field of proteins under the guidance of Emil Fischer and is probably the most prolific research worker in the field of proteins. Since 1904 he has published literally hundreds of papers dealing with one or another phase of protein chemistry, in addition to many papers in other fields. No consideration of workers in the field of proteins would be complete without the acknowledgment of the advances which have been made by Abderhalden and his students.

As already noted, Ritthausen early began an investigation of the vegetable proteins. Owing to the lack of organized technic and any definite knowledge of protein structure, Ritthausen's contributions, while important, were inadequate in this field.

Thomas B. Osborne began work in the field of vegetable proteins about 1895 and from that time until his death in 1929 has been recognized as the outstanding authority in this field of protein research. It is to Osborne that we owe many improvements in the methods of protein analysis and much of our information in regard to the methods of isolation and purification of the vegetable proteins.

During the nineteenth century, organic chemistry dominated chemical science. Toward the end of the nineteenth century, however, physical chemistry began more and more to assume a supremacy. It is not surprising, therefore, that many of the more important recent contributions in the field of protein study should be characterized by the application of physico-chemical methods to this field. The author feels that the outstanding contribution to a physico-chemical study of the proteins is the contribution which has been made by Sörensen.[35] These papers of Sörensen will be referred to more extensively in the following pages.

THE REACTIVE GROUPS IN PROTEINS.—In all of his studies on the proteins Fischer has emphasized the reactions which take place between a primary amino group and the carboxyl group of an organic acid. In his synthesis of the polypeptides, he has given us the peptide linkage,

$$R—NH_2 + R'—COOH = R—NH—COR' + H_2O,$$

[35] Sörensen, S. P. L., Studies on Proteins, *Compt. rend. Lab. Carlsberg*, Vol. 12 (1917).

and it has been definitely proven that the peptide linkage occurs in the unaltered proteins. Probably due to the fact that Fischer emphasized this particular linkage, and due to the fact that Fischer's personality and research genius were so great, attention has been focussed by research workers almost entirely upon the amino and the carboxyl groups of the proteins, in an attempt to explain protein structure and protein reactions. There is a certain amount of definite evidence, however, that while the amino groups and carboxyl groups are of primary importance, other groups, and linkages other than the peptide linkage may and probably do occur in the unaltered proteins. A consideration of the reactive groups present in the various amino acids should indicate to any organic chemist the possibility of other reactions and other linkages. The following groups are present in at least one of the amino acids noted in Table XLV:

1. Primary amino group ($—NH_2$).
2. Carboxyl group (—COOH), especially in the di-carboxylic acids.
3. Aliphatic alcohol group (—OH).
4. Phenolic group (—OH), (aromatic alcohols).
5. Alcohol group intermediate between the aliphatic (—OH) and the aromatic (—OH), as the (—OH) in oxyproline.
6. Imino group (=NH).
7. Acid amide group ($—CO—NH_2$).
8. Sulfhydryl group (—SH) in cysteine, or the disulfide group (—S—S—) of cystine.
9. α-Hydrogen of tryptophane.
10. The guanidine nucleus.

That certain of these groups are reactive is evidenced by the analyses of Skraup and Krause[36] who report 0.85 per cent of methoxy ($—OCH_3$) groups and 1.13 per cent of N-methyl ($—NH—CH_3$) or ($—N(CH_3)_2$) groups in casein, and the analyses of Geake and Nierenstein[37] who report that the unaltered casein contains from 0.49 to 0.55 per cent of methoxy groups and from 0.87 to 0.88 per cent of N-methyl groups. Herzig and Landsteiner[38] report 0.64 per cent of methoxy and 1.78 per

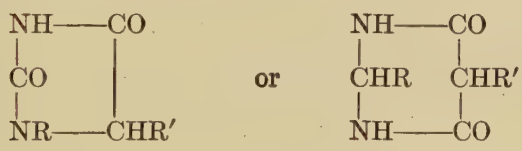
[36] Skraup, Z. H., and Krause, E., Über die Einwirkung von Jodmethyl auf Casein, *Monatsch. f. Chem.*, 30 : 447–465 (1909).

[37] Geake, A., and Nierenstein, M., The Action of Diazomethane on Caseinogen, *Biochem. J.*, 8 : 287–292 (1914).

[38] Herzig, J., and Landsteiner, K., Über die Methylierung von Eiweissstoffen, *Biochem. Z.*, 61 : 458–463 (1914).

cent of N-methyl groups in casein. Accordingly there appears to be fairly adequate evidence for the existence of methoxy and N-methyl derivatives in the unaltered protein, but no compound containing these groups has ever been isolated from protein material. Theoretically the ether linkage is a possibility. The presence of such a linkage, except in the case of thyroxine, still remains to be proven. However, inasmuch as other biological compounds are known, in which the ether structure exists, that are highly methylated or ethylated, we must conclude that the biological organism possesses the mechanism for forming such compounds and that probably our inadequate methods of protein study are responsible for the fact that they have as yet been unidentified among the protein decomposition products.

Dunn[39] points out that there is a liberation of carbon dioxide during the acid hydrolysis of a protein, amounting in the case of casein to 0.71 per cent of the weight of the casein. He suggests that the sources of this carbon dioxide may be uramino acids, hydantoins, or cyclic diacipiperazines, possibly in such structures as:



The Synthesis of Amino Acids.—Four general types of reactions have been utilized for the synthesis of α-amino acids.

1. The synthesis of an α-amino acid from an *α-halogen acid* and ammonia,

$$R—CH(Cl)—COOH + NH_3 = R—CH(NH_2)—COOH + HCl$$

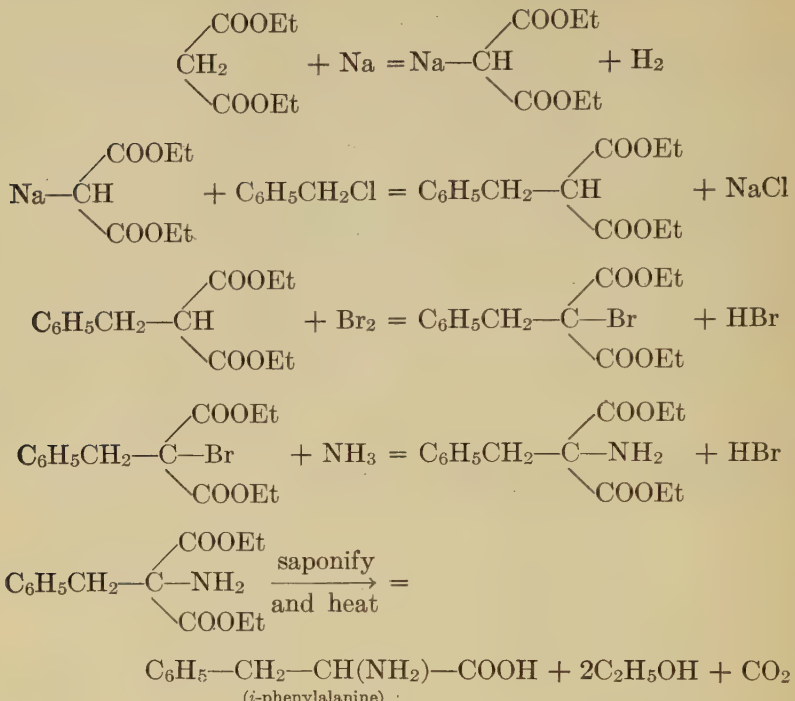
This reaction, as a rule, works smoothly, the only difficulty being the securing of the proper halogen acid.

2. The synthesis from an *aldehyde* having one less carbon than the desired amino acid, by the addition of hydrocyanic acid and ammonia, with the subsequent saponification of the nitrile,

$$R—CHO + HCN = R—CH(OH)CN$$
$$R—CH(OH)CN + NH_3 = R—CH(NH_2)CN + H_2O$$
$$R—CH(NH_2)CN + 2H_2O = R—CH(NH_2)—COOH + NH_3$$

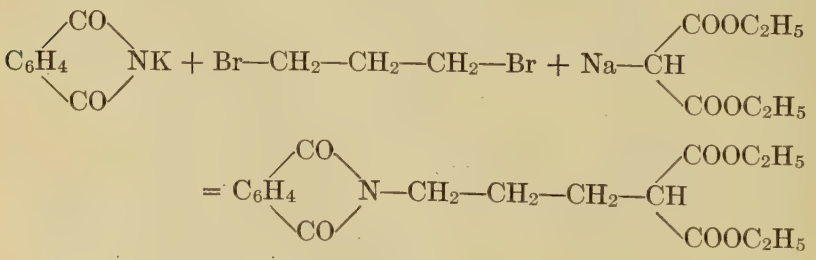
[39] Dunn, M. S., The Liberation of Carbon Dioxide, Ammonia, and Amino Nitrogen from Casein by Acid Hydrolysis, *J. Am. Chem. Soc.*, 47 : 2564–2568 (1925).

3. The synthesis from *malonic ester* through the halogen compound



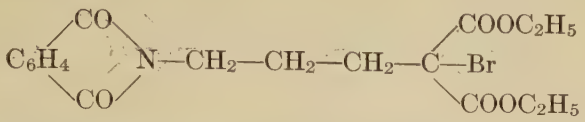
The above series of reactions was carried out by Fischer in the synthesis of phenylalanine. Any aryl or alkyl radical may be substituted for the $C_6H_5CH_2$— radical of the benzyl chloride.

4. The synthesis from phthalimide. This synthesis was used by Fischer[40] in the preparation of ornithine. Potassium phthalimide, propylene bromide and sodium malonic ester were combined to form γ-phthalimidopropyl malonic ester.

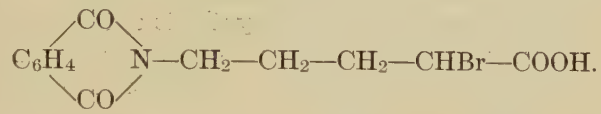


[40] Fischer, E., Synthese der α-δ-di-aminovaleriansäure, *Ber.*, 34 : 454–464 (1901).

The γ-phthalimidopropyl malonic ester was then treated with bromine to form γ-phthalimidopropyl brom malonic ester,



This compound was then saponified to the substituted malonic acid and this by heating lost carbon dioxide, yielding δ-phthalimido α-brom valerianic acid,

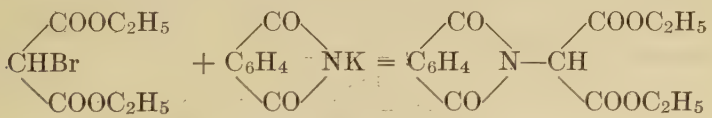


On treating this with ammonia, the bromine was replaced with ($—NH_2$) and hydrolysis with hydrochloric acid then split off phthalic acid and yielded inactive *ornithine*, α-δ-di-amino-valerianic acid,

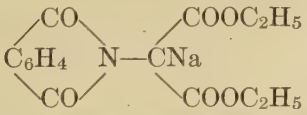
$$NH_2—CH_2—CH_2—CH_2—CH(NH_2)—COOH.$$

The substituted brom malonic acid could not be directly converted into an amino compound, treatment with ammonia resulting in the formation of cyclic compounds. It was accordingly necessary to add the ammonia to the substituted brom valerianic acid.

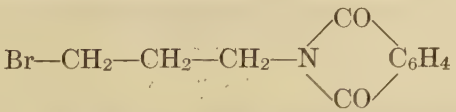
Sörensen[41] used phthalimide to add both the α and δ amino groups. Brom malonic ester was combined with phthalimide potassium to form phthalimido malonic ester,



This compound was converted into phthalimido sodium malonic ester,

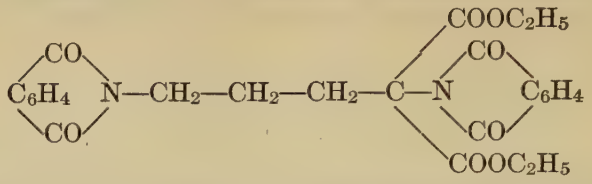


which was combined with γ-brom propyl phthalimide,



[41] Sörensen, S. P. L., Études sur la synthèse des acides amidés, *Compt. rend. Lab. Carlsberg*, 6 : 1–60 (1903).

yielding γ-phthalimido propyl phthalimido malonic ester,



The ester groups were split off by saponification, the free acid lost carbon dioxide on heating, and the phthalic acid residues were removed by acid hydrolysis, forming the desired *ornithine* in good yield.

The methods that are used for the synthesis of the oxy amino acids are dependent very largely upon the particular amino acid which it is desired to synthesize. Only a single illustration will be given, *i.e.*, Leuchs and Geiger's[42] synthesis of serine. The starting material for this synthesis is chloracetal which is heated with sodium ethylate in an autoclave, yielding ethoxyacetal, this being hydrolyzed to ethoxyacetaldhyde, the amino acid being formed through the cyanhydrin synthesis, as indicated by the following reactions:

$$Cl—CH_2—CH—(O—C_2H_5)_2 + NaOEt = Et—O—CH_2—CH—(O—C_2H_5)_2 + NaCl$$

$$Et—O—CH_2—CH—(O—C_2H_5)_2 + H_2SO_4 = Et—O—CH_2—CHO + 2C_2H_5OH$$

$$Et—O—CH_2—CHO + HCN = Et—O—CH_2—CH(OH)CN$$

$$Et—O—CH_2—CH(OH)CN + NH_3 = Et—O—CH_2—CH(NH_2)CN + H_2O$$

$$Et—O—CH_2—CH(NH_2)CN \xrightarrow[\text{saponify}]{} Et—O—CH_2—CH(NH_2)COOH$$

$$Et—O—CH_2—CH(NH_2)COOH \xrightarrow{\text{conc. HBr}} OH—CH_2—CH(NH_2)COOH$$

(*i* serine)
(β-oxy-α-amino propionic acid)

The above reactions are capable of producing a fair yield of inactive serine. In the author's laboratory, starting with 200 grams of chloracetal, approximately 20 grams of pure inactive serine was obtained.

The Separation of the Racemic Mixture into Its Active Components.—With the exception of glycine (amino acetic acid), all of the naturally-occurring amino acids, contain an asymmetric carbon atom.

[42] Leuchs, H., and Geiger, W., Ueber eine neue Synthese des Serins, *Ber.*, 39 : 2644–2649 (1906).

As is usual in biological compounds, the naturally-occurring amino acids are optically active, only the *dextro* or the *levo* form occurring in the protein. It is only in rare instances that asymmetric synthesis occurs in the synthesis of organic compounds. Almost invariably, compounds synthesized in the organic laboratory are optically inactive, and are what is known as a *racemic mixture*, where equal quantities of the *d* and *l* forms are present. Such a mixture is optically inactive, the levo-rotation of the *l* form being neutralized by the dextro-rotation of the *d* form.

As we shall see later, there may be pronounced differences in solubility and in physiological action between the *d* and *l* forms of a chemical compound. Accordingly the task of a biological chemist is not completed when one of the syntheses noted above has yielded the pure amino acid. He must still separate the racemic mixture into its optically active components.

Three general methods are available for such separation.

1. *Mechanical Separation.*—Since the crystals of the *d* and *l* forms are mirror images of each other, it is possible to pick out the different crystals from a crystal mixture by hand, using forceps under a lens. Pasteur used this method to separate *d* and *l* tartaric acids. Unfortunately, amino acids rarely or never crystallize in large enough crystals to allow one to use this method of separation, so that the mechanical separation is of practically no use in protein research.

2. *The Biological Method.*—Biological organisms show a surprisingly high degree of specificity toward organic molecules. The yeasts, molds, and bacteria, as well as the higher animals, are usually capable of utilizing only one form of an optically active amino acid. In general, the optically active form which occurs in proteins is the one which is attacked by the biological organism.

In utilizing the biological method, the mixture of amino acids is inoculated with a pure culture of a bacterium, a fungus, or a yeast, in a culture medium, and the organism is allowed to grow and develop until one optically active isomer has been completely destroyed. The solution is then worked up for the isolation of the optically active isomer which was not attacked by the biological organism. Unfortunately, while this method yields one of the optically active isomers, it is usually the optically active isomer which does not occur in nature. Accordingly, the biological method is rarely of great value in the isolation of the naturally-occurring compound, that compound having been destroyed by the microorganism.

3. *The Chemical Method.*—The chemical method for separating racemic mixtures of amino acids depends upon the formation of a compound of the amino acid with some optically active substance. Alka-

loids are very generally used for this purpose, inasmuch as they can be obtained in quantity and a high degree of purity at a comparatively low cost. The alkaloids, quinine, strychnine, brucine, and cinchonine, are the alkaloids usually employed, inasmuch as they can be readily crystallized and purified. The alkaloids possess pronounced basic properties (*vide infra*), but the amino acids are not sufficiently acidic to combine directly with the alkaloids. It is accordingly necessary to intensify the acidic properties of the amino acid molecule. This is usually done by forming the benzoyl derivative by the addition of benzoyl chloride to an amino acid.

$$R{-}CH(NH_2){-}COOH + C_6H_5COCl = R{-}CH(NHCOC_6H_5)COOH + HCl.$$

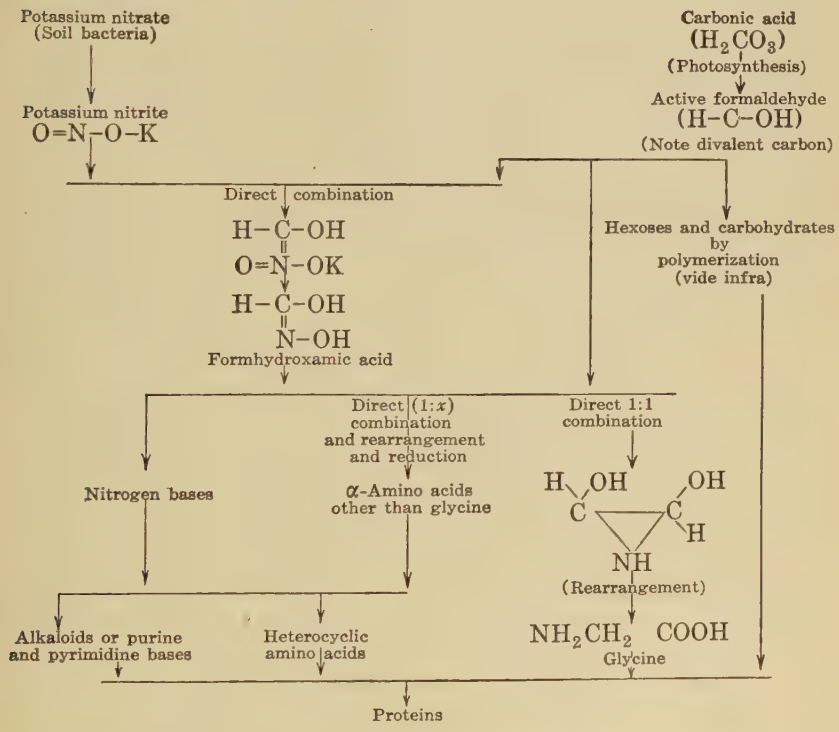
In this way the basicity of the amino group is masked, the acidity of the benzoyl derivative being intensified so that it forms a salt with the basic alkaloid.

The alkaloid which is selected and the benzoyl derivative are then mixed in the stoichiometrical proportions necessary for the formation of the alkaloid salt. Assuming that strychnine is the alkaloid chosen and that racemic phenylalanine is the amino acid which it is desired to separate into the optically active isomers, we would have a mixture of *l*-strychnine-*l*-benzoyl-phenylalanine and *l*-strychnine-*d*-benzoyl-phenylalanine. Such compounds will be found to differ in solubility. In some instances this difference is only slight. In other instances it may be very appreciable. The mixture is accordingly separated by a series of fractional crystallizations, retaining at one end of a series the most insoluble fraction and at the other end of the series the most soluble fraction. After a series of fractional crystallizations, testing the extreme fractions for the rotation of polarized light in a polarimeter, it will usually be found that one end of the series yields a constant dextro-rotation, the other end of the series a constant levo-rotation, indicating that a complete separation has been obtained. When the separation is complete, the alkaloidal base is removed by appropriate means, the benzoyl derivative is hydrolyzed off by acid, and the optically active amino acid separated and recrystallized until it shows constant physical properties. If the crystals and the physical and chemical properties of an amino acid so prepared are identical with the amino acid prepared from protein material, then and only then can one state with certainty that the naturally occurring amino acid has been synthesized.

The Synthesis of Amino Acids in Nature.—Practically nothing is known in regard to the mechanism whereby amino acids are synthesized in the plant cell. As we shall see later, the entire animal kingdom is

dependent upon the plant for the production of amino acids. *With the possible exception of glycine, the animal body or at least the tissues of the higher animals are incapable of synthesizing any single amino acid in quantities adequate for growth or maintenance.* The animal is accordingly dependent upon the plant for the amino acids which go to make up the muscle and vital proteins of the animal body.

The synthesis of amino acids and proteins in the plant is a part of the photosynthetic process. We know that carbon dioxide, water, and nitrates or nitrites are the ultimate sources from which the plant synthesizes the amino acids. Baly[43-44] has suggested a mechanism whereby amino acids may be synthesized from carbon dioxide, water, and nitrates. His scheme is illustrated in the following diagram.



[43] Baly, E. C. C., Heilbron, I. M., and Hudson, D. P., Photocatalysis, Pt. II. The Photosynthesis of Nitrogen Compounds from Nitrates and Carbon Dioxide, *J. Chem. Soc.*, London, 121 : 1078–1088 (1922).

[44] Baly, E. C. C., Heilbron, I. M., and Stern, H. J., Photocatalysis, Pt. III. The Photosynthesis of Naturally Occurring Nitrogen Compounds from Carbon Dioxide and Ammonia, *J. Chem. Soc.*, London, 123 : 185–197 (1923).

He has presented certain evidence in favor of these theories and reports color reactions in artificial photosynthetic studies, indicating that α-amino acids in small quantities have been formed in his experiments. He also reports the synthesis of the alkaloid, coniine, in his artificial photosynthetic studies. Baly's scheme, as noted in the diagram above, appears to be the most logical scheme which has as yet been presented to account for the various nitrogenous constituents which are formed by vital processes. However, much additional experimental evidence is needed before we can accept this scheme as representing the reactions which actually occur in the vital syntheses.

CHAPTER XII

POLYPEPTIDES

The name, polypeptides, was given by Fischer to compounds of two or more amino acids joined together by the peptide linkage. Most of our knowledge of polypeptides is due to the work of Emil Fischer or Emil Abderhalden. Polypeptides have been prepared by two general methods: (1) the partial hydrolysis of proteins, following some such procedure as allowing a protein to stand 12 to 48 hours in contact with *cold*, 60–80 per cent sulfuric acid, diluting the mixture with water, taking care that no appreciable rise in temperature occurs, neutralizing the sulfuric acid with barium hydroxide, again guarding against elevated temperature, filtering off the barium sulfate, and fractionally crystallizing the partial hydrolysis products in an attempt to isolate definite chemical compounds which could be later characterized insofar as amino-acid content is concerned, and (2) the synthesis of polypeptides of known structure by the condensation of amino acids or amino-acid derivatives.

The Synthesis of Polypeptides.—Schaal, in 1871, described an "anhydride" of aspartic acid, and Grimaux, in 1882, reported that this anhydride spontaneously transformed itself into "colloidal polyaspartic urea." Schiff, 1898–1899, on further investigation named the product, polyaspartic acid. At about the same time, Schützenberger in a series of studies on "the synthesis of albuminoses and protein materials" treated various amino acids with phosphorus pentoxide and caused the formation of complex substances. Lilienfeld, in 1894, obtained complexes by treating amino-acid mixtures with various condensing agents, such as potassium pyrosulfate, formaldehyde, etc., and Balbiano and Frasciatti, in 1900, converted glycine into a horn-like substance by heating it with glycerol.

All the products obtained by the above workers were amorphous materials, having indifferent chemical characteristics, such that it was impossible to classify them or to determine their true relationship to the proteins.

Fischer early pointed out that in order to arrive at a definite conclu-

sion, some method must be devised whereby one could build up or tear down a chain of amino acids at will and that the reactions must be so controlled that the various steps of the process could be traced by the usual procedures of the organic laboratory.

Previous to Fischer, Curtius had busied himself with linking amino acids together, but in practically every instance he had used not the free amino acid but the benzoyl derivative. In his studies, he obtained definite crystalline organic compounds. These, however, were very different from the true polypeptides which were later to be synthesized, because of the fact that Curtius' compounds contained the benzoyl radical.

As early as 1882, Curtius treated glycine silver with benzoyl chloride and besides obtaining hippuric acid, C_6H_4—CO—NH—CH_2—COOH (benzoyl-amino-acetic-acid), he obtained a substance which he called "hippuryl-amino-acetic-acid" and which Fischer later showed to be the benzoyl derivative of the polypeptide, glycyl-glycine,

$$C_6H_4\text{—}CO\text{—}NH\text{—}CH_2\text{—}CO\text{—}NH\text{—}CH_2\text{—}COOH.$$

Curtius likewise obtained a second compound, which he called "γ acid," by the union of hippuric acid ester and glycine. This compound gave the biuret reactions, although the color was somewhat different from the biuret reaction as given by unaltered protein. Twenty-one years later, in the light afforded by Fischer's studies, Curtius repeated his experiments and found that the "γ acid" was in reality benzoyl-pentaglycyl-glycine. As early as 1884, Curtius had stated that besides hippuric acid a number of compounds could be obtained from glycine silver and benzoyl chloride, each compound differing from the other by one molecule of glycine with the elimination of one molecule of water. Accordingly Curtius must be credited with the first suggestion as to the peptide linkage and with the theoretical possibilities of linking amino acids together in chains to form polypeptides. Fischer pointed out that while Curtius' statements were excellent theory, his experimental facts to prove the theory were lacking, except in the instances noted above.

Curtius observed another phenomenon, *i.e.*, that glycine ester in water solution could be transformed into glycine anhydride or diketopiperazine, the simplest member of the diketopiperazines,

$$2NH_2\text{—}CH_2\text{—}COOC_2H_5 \longrightarrow \begin{matrix} NH\text{—}CO\text{—}CH_2 \\ | \qquad\qquad | \\ CH_2\text{—}CO\text{—}\ NH \end{matrix} + 2C_2H_5OH$$

(Diketo piperazine)

In 1901, Fischer and Fourneau[1] hydrolyzed the glycine anhydride with acids and obtained the first polypeptide, *glycyl-glycine.* In a study of the various derivatives of glycyl-glycine, Fischer prepared the ethyl ester, and the carbethoxy ester of glycyl-glycine-ester by the action of ethyl chlor carbonate,

$$C_2H_5—O—CO—Cl + NH_2—CH_2—CO—NH—CH_2—COOC_2H_5 =$$
$$C_2H_5—O—CO—NH—CH_2—CO—NH—CH_2—COOC_2H_5$$

(Carbethoxy-glycyl-glycine-ester)

A few months later he found that this carbethoxy ester would unite with the ester of another amino acid, *e.g.*, leucine ester, to give carbethoxy-glycyl-glycine-leucine-ester,

$$C_2H_5—O—CO—NH—CH_2—CO—NH—CH_2—CO—NH—CH(C_4H_9)CO—O—C_2H_5$$

the carbethoxy group of the glycyl-glycine-ester intensifying the acidic properties of the compound, so that it unites directly with the leucine ester, with the elimination of a molecule of ethyl alcohol. At the same time Fischer announced a second polypeptide, leucyl-leucine, prepared by the partial hydrolysis of leucineimide, which itself had been described more than fifty years before.

Some months later, Curtius (1902) announced that by treating glycine with hippurylazide ($C_6H_5—CO—NH—CH_2—CO—N_3$) he could add one glycyl radical, and by repetition of the process he could lengthen the chain up to benzoyl-pentaglycyl-glycine. Thus, both Fischer and Curtius devised methods whereby chains of amino acids could be built up. In the case of Fischer's method, however, the resulting compounds contained the carbethoxy group, and in the case of Curtius' method, the benzoyl radical. Accordingly the resulting compounds did not show physical properties similar to those possessed by the partial hydrolysis products of proteins.

In 1903, Fischer for the first time was able to prepare the *acid chloride* of glycine derivatives by treating carbethoxy-glycine with thionyl chloride. This acid chloride would condense with an amino-acid ester to form a carbethoxy-dipeptide, which in turn could be converted into the acid chloride and further condensed with another molecule of an amino-acid ester. He was able to saponify the ester group of the peptides so formed, and obtained carbamino-tri-glycyl-glycine, having presumably the formula,

$$HOOC—NH—CH_2—CO—NH—CH_2—CO—NH—CH_2—COOH.$$

[1] Fischer, E., and Fourneau, E., Über einige Derivate des Glykocolls, *Ber.*, 34 : 2868–2877 (1901).

This compound was nearer to a true tripeptide than were the benzoyl derivatives prepared by Curtius, or the carbethoxy derivatives prepared by Fischer, but still possessed a carboxyl group which could not be removed.

Accordingly, Fischer again altered his technic. Using chloracetyl chloride and glycyl-glycine-ester, he obtained chloracetyl-glycyl-glycine,

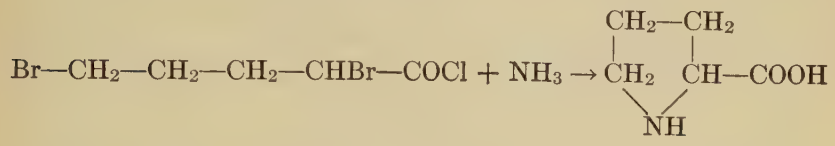
$$Cl—CH_2—CO—Cl + NH_2—CH_2—CO—NH—CH_2—CO—O—C_2H_5$$
$$= Cl—CH_2—CO—NH—CH_2—CO—NH—CH_2—CO—O—C_2H_5,$$

and on treating this compound with ammonia the chlorine was replaced with $—NH_2$, yielding the tripeptide, di-glycyl-glycine. This compound he esterified and prepared the benzoyl derivative which was identical with a benzoyl product that Curtius had synthesized several years before.

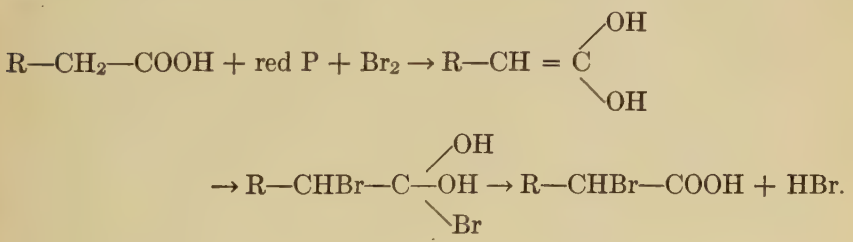
In his study of the synthesis of amino acids, Fischer prepared a number of α-brom acids which could be easily converted into the corresponding acid chlorides by treatment with PCl_5. These acid chlorides he found would combine with amino acid esters to form derivatives containing bromine, and when the bromine was removed by treatment with ammonia, polypeptides resulted. Thus, α-brom-isocaproyl-chloride will combine with glycyl-glycine-ester to form α-brom-isocaproyl-glycyl-glycine-ester, which on treatment with ammonia and saponification of the ester group, is converted into leucyl-glycyl-glycine. *Fischer further found that those derivatives of the polypeptides in which a halogen-containing acyl radical was attached to the amino group could be converted into the corresponding acid chloride by treatment with* PCl_5. Thus, α-brom-isocaproyl-glycyl-glycine could be converted by PCl_5 into α-brom-isocaproyl-glycyl-glycine-chloride, and this in turn could be condensed with glycyl-glycine-ester to form α-brom-isocaproyl-triglycyl-glycine-ester, and when this compound was treated with ammonia, the bromine was replaced with $—NH_2$, resulting in a pentapeptide, leucyl-triglycyl-glycine; or prior to the treatment with ammonia, the compound could be again converted into an acid chloride and further condensations carried out. *It is impossible to prepare the acid chloride of an unsubstituted amino acid or of an unsubstituted polypeptide.* The acid chlorides can, however, be obtained, providing the amino group is masked with such radicals as carbethoxy, benzoyl, etc., or with radicals such as brom-isocaproyl, brom-phenylacetyl, etc. It is obvious, of course, that if one is to prepare derivatives corresponding to the natural amino acids of proteins, the halogen must be in the α position.

Fischer prepared a number of α-halogen acids for use in his polypeptide syntheses. Thus,

Brom acetyl chloride treated with ammonia yields glycine.
α-brom propionyl chloride yields alanine.
α-brom butyryl chloride yields α-amino butyric acid.
α-brom isocaproyl chloride yields leucine.
α-brom phenyl acetyl chloride yields α-amino-phenyl acetic acid.
α-brom phenyl hydrocinnamyl chloride yields phenylalanine.
α-δ-dibrom-valeryl-chloride, instead of yielding an α-δ-dibrom-amino acid, condenses to form proline.



The difficulty with using α-halogen acids either to synthesize amino acids or as an aid in the synthesis of polypeptides, has in the past been the difficulty of securing adequate amounts of a desired halogen acid. Ward [2] has shown that red phosphorus catalyzes the bromination of aliphatic acids in the α-position and suggests that this may be due to the following reactions:



Accordingly, using red phosphorous as a catalyst in the bromination procedure, it should be possible to very greatly speed up research work involving the use of considerable quantities of amino acids.

Summarizing the methods for the synthesis of polypeptides, we find that they can be divided into three groups, all due to the research activities of Emil Fischer:

1. The splitting of amino acid anhydrides or diketopiperazines by mild acid hydrolysis.
2. Condensing chloracetyl chloride with amino acid esters or esters of polypeptides and later treating the derivatives, so obtained,

[2] Ward, C. F., The Bromination of Acids in the α-Position, *J. Chem. Soc.*, London, 121 : 1161–1165 (1922).

with ammonia in order to convert the chloracetyl radical into a glycyl radical.

3. Forming acid chlorides of polypeptide derivatives which still contain in the molecule a halogenated acyl radical.

By use of this last process, Fischer,[3] in 1907, prepared an octadecapeptide (18 amino acids), leucyl-triglycyl-leucyl-triglycyl-leucyl-octaglycyl-glycine. He prepared this by combining brom-isocaproyl-diglycyl-glycine-chloride with pentaglycyl-glycine, yielding, when treated with ammonia, leucyl-octaglycyl-glycine. This was further combined with another molecule of brom-isocaproyl-diglycyl-glycine-chloride, yielding, when treated with ammonia, leucyl-triglycyl-leucyl-octaglycyl-glycine. A further treatment with an additional molecule of brom-isocaproyl-diglycyl-glycine-chloride yielded, when the product was treated with ammonia, the octadecapeptide. This compound for many years had the highest molecular weight (1,213) of any compound prepared synthetically. Later Abderhalden and Fodor[4] prepared a polypeptide containing 19 amino acids, *l*-leucyl-triglycyl-*l*-leucyl-triglycyl-*l*-leucyl-triglycyl-*l*-leucyl-pentaglycyl-glycine, with a molecular weight of 1,326. If either one of the above polypeptides had contained amino acids other than glycine, such as tyrosine or phenylalanine, the molecular weight would have been about 3,000. Fischer describes his compound as a colorless, amorphous powder, difficultly soluble in hot water to a solution which becomes turbid on standing; the solution foams, later is precipitated by saturation with ammonium sulfate, precipitated by tannins and by phosphotungstic acid, it gives the biuret test, but as would be expected, no other characteristic color tests. The turbidity in aqueous solution and its ease of foaming indicate that it is approaching colloidal dimensions, if indeed the solution is not a typical colloidal sol.

Abderhalden has listed in a table certain of the physical properties of his nonadecapeptide, together with the changes in physical properties of the various intermediate products. His findings are reproduced in Table XLVI.

It will be noted that the solubility is greatly decreased as the length of the polypeptide chain is increased, and that the polypeptide becomes more and more readily salted out from solution by saturation with ammonium sulfate. Likewise, the color of the biuret reaction changes with increasing length of the carbon chain.

[3] Fischer, E., Synthese von Polypeptiden. XVII., *Ber.* 40 : 1754–1767 (1907).

[4] Abderhalden, E., and Fodor, A., Synthese von hochmolekularen Polypeptiden aus Glykokoll und *l*-Leucin, *Ber.*, 49 : 561–578 (1916).

TABLE XLVI

Properties of Polypeptides with Increasing Lengths of Polypeptide Chains

(Data of Abderhalden)

Number of Amino Acids in the Compound	Polypeptide	Solvent for Determining Specific Rotation	Optical Rotation α_D^{20}	Biuret Reaction	Behavior Toward Saturation of Solution with Ammonium Sulfate	Solubility in Cold Water
3	*l*-leucyl-glycyl-glycine	Water	+45.90°	Blue-violet	Not salted out	Very easily
5	*l*-leucyl-triglycyl-glycine	Water	+28.14°	Blue-violet	Not salted out	Easily
7	*l*-leucyl-pentaglycyl-glycine	N/10 NaOH	+ 5.94°	Red-violet	Salted out	Fairly easily
11	*l*-leucyl-triglycyl-*l*-leucyl-pentaglycyl-glycine	N/10 NaOH	− 6.00°	Red-violet	Salted out	Difficultly
15	*l* - leucyl - triglycyl - *l* - leucyl-triglycyl - *l* - leucyl - pentaglycyl-glycine	N/10 NaOH	− 9.63°	Red-violet	Salted out	Difficultly
19	*l* - leucyl - triglycyl - *l* - leucyl-triglycyl - *l* - leucyl - triglycyl - *l*-leucylpentaglycyl-glycine	N/10 NaOH	− 8.42°	Red-violet	Salted out	Difficultly

The Linkage in Polypeptides.—There are four possible structural formulae for a simple polypeptide, such as glycyl-glycine.

1. $NH_2—CH_2—CO—NH—CH_2—COOH$

2. $NH_3—CH_2—CO—NH—CH_2—CO$
   $\quad\quad\quad

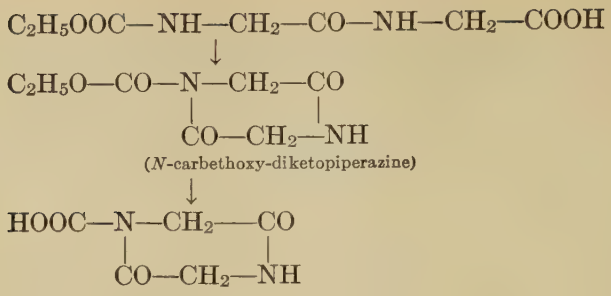
groups of guanidine do not react to form carbamino acids. Siegfried and Liebermann[5] found that polypeptides also gave the carbamino reaction. Dipeptides, however, gave a ratio varying from 1.63 : 1 to 1.79 : 1, tripeptides a ratio of approximately 2.57 : 1, and tetrapeptides a ratio of 3.29 : 1 instead of the expected 2 : 1, 3 : 1, and 4 : 1 ratios, showing that some group other than the single, free amino group was functioning to some extent.

It should be noted at this point that this carbamino reaction is of importance in laboratory technic. Proteins, peptones, and amino acids, all undergo the addition of carbon dioxide in alkaline solution, with the formation of carbamino acid derivatives. Accordingly, if one hydrolyzes the protein with sulfuric acid, adds an excess of barium hydroxide to remove the sulfuric acid, and then bubbles carbon dioxide through the solution to precipitate barium carbonate, it invariably happens that very appreciable amounts of barium are retained in solution, due to the formation of soluble barium salts of the carbamino acids. When such a solution is boiled, a part of the carbamino acid breaks down, precipitating barium carbonate. This reaction, however, does not immediately go to completion. Accordingly, it is very difficult to free a solution, containing proteins, peptones, or amino acids, from barium by converting the barium into barium carbonate. Barium will often remain in the final concentrates and will have to be removed by adding an equivalent of sulfuric acid.

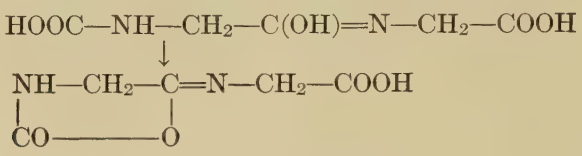
Leuchs and Manasse[6] in a study of this reaction noted that carbethoxy-glycyl-glycine-ester prepared from ethyl-chlor-carbonate and glycine ester, when hydrolyzed would not yield carbamino-glycyl-glycine-ester as expected, but instead decomposed into glycine and carbon dioxide. They did, however, obtain from carbethoxy-glycyl-glycine-chloride an anhydride which formed a barium salt which yielded an anhydride of glycine, having different properties from the diketopiperazine prepared by Fischer, and which was isomeric with Fischer's diketopiperazine. On the hydrolysis of this anhydride, instead of obtaining the stable glycyl-glycine-carboxylic-acid of Fischer, they obtained a very unstable compound. They represent the two isomeric acids and isomeric ring structures as follows; the *unstable* form of ring structure being derived from the acid ester,

[5] Siegfried, M., and Liebermann, H., Über die Bindung von Kohlensäure durch amphotere Aminokörper, *Z. physiol. Chem.*, 54 : 437–447 (1908).

[6] Leuchs, H., and Manasse, W., Über die Isomerie der Carbäthoxyl-glycyl-glycinester, *Ber.*, 40 : 3235–3249 (1907). (Cf. also Leuchs, H., *Ber.*, 39 : 857–861 (1906); Leuchs, H., and Geiger, W., *Ber.*, 41 : 1721–1726 (1908); and Leuchs, H., and La Forge, F. B., *Ber.*, 41 : 2586–2596 (1908)).



which at once loses carbon dioxide and goes to diketopiperazine. The *stable* form of ring structure is derived from the acid,



a staple compound which does not lose carbon dioxide.

It will be noted that the stable ring compound is a *lactone*. Therefore, the stable form of glycyl-glycine-carbamino-acid contains the grouping —N=COH— and is a *lactim* derivative, whereas the unstable form contains the grouping —NH—CO— and is a *lactam* derivative. The formulae of polypeptides are usually written in the lactam form. Inasmuch, however, as this form permits the addition of carbon dioxide and the formation of carbamino acids, whereas the lactim does not, it would appear that in the light of the N : CO_2 ratios noted above, probably both the lactam and the lactim groupings occur in polypeptides. This is in agreement with the argument of Robertson[7] for a keto $\rightleftharpoons$ enol isomerism of the peptide linkage in proteins.

Polypeptides from Proteins.—As already noted, a number of workers have partially hydrolyzed proteins and have isolated polypeptides from the decomposition products. In a number of instances the amino acids present have been identified and their quantities accurately determined. Assuming that the product isolated is a tripeptide containing tyrosine, leucine, and alanine, there are six possible isomeric compounds having identical composition, *i.e.*,

1. Tyrosyl-leucyl-alanine.
2. Leucyl-tyrosyl-alanine.
3. Alanyl-leucyl-tyrosine.

[7] Robertson, T. B., The Physical Chemistry of the Proteins, Longmans, Green and Company, New York (1918).

4. Leucyl-alanyl-tyrosine.
5. Alanyl-tyrosyl-leucine.
6. Tyrosyl-alanyl-leucine.

Accordingly, the identification of a naturally occurring polypeptide or of a polypeptide isolated from protein decomposition products must be made, in the last analysis, by synthesizing the various possible isomers and by comparing the physical and chemical properties of the resulting synthetic products with the polypeptide which was isolated. In a number of instances this has been done, notably in the laboratories of Fischer and Abderhalden.

The Value of Polypeptide Studies.—The study of polypeptides has thrown much light upon protein structure.

1. Inasmuch as the synthetic polypeptides have in some instances been shown to be identical with the polypeptides isolated by the partial hydrolysis of proteins, we know definitely that the —NHCO— or —N=COH— group is present in the proteins. This fact, however, does not prove that the peptide linkage is the only linkage in proteins. In fact, polypeptide study indicates very strongly that it is not the only linkage, and while certain of the synthetic polypeptides are hydrolyzed by tryptic enzymes, none, insofar as the author is aware, are hydrolyzed by the peptic enzymes. Fischer concluded that the chains of the polypeptides were not long enough for pepsin to act upon them, but it seems more probable that pepsin attacks some linkage other than the linkage in the peptide group. Trypsin hydrolyzes proteins to their constituent amino acids. Pepsin hydrolyzes proteins only to proteoses, peptones, and polypeptides.

2. The behavior of polypeptides toward tryptic enzymes has shown in a striking manner the specificity of enzyme action. Trypsin hydrolyzes certain polypeptides; others it does not attack. In a racemic mixture of carbethoxy-glycyl-*d*-*l*-leucine, it hydrolyzes the polypeptide of which *l*-leucine is a component but does not attack the polypeptide containing *d*-leucine. Alanyl-glycine is hydrolyzed by trypsin; glycyl-alanine is not. The position of the amino acid in the molecule has a marked influence on the ease of tryptic hydrolysis. When alanine is the acyl radical with glycine, alanine or leucine, hydrolysis occurs, but when leucine, valine, or aminobutyric were the acyl radicals with alanine, no hydrolysis took place. If the free carboxyl group is attached to tyrosine, cystine, or isoserine, they are readily split off by trypsin. In at least one instance examined by Fischer, where tyrosine was the acyl radical, no hydrolysis occurred.

The number of amino acids in the chain influences tryptic action.

Triglycyl-glycine was not attacked by trypsin. Tetraglycyl-glycine was hydrolyzed. Triglycyl-glycine-ester was likewise hydrolyzed.

Natural proteins to some extent show similar differences. Fibrin, for example, is very easily digested by pepsin. Edestin (from hemp seed) is very slowly attacked. On the other hand, edestin is more easily attacked by trypsin than is fibrin.

Abderhalden has studied the effect of other enzymes than those of the pancreas, on many polypeptides. The enzymes which he studied were nearly all " tryptic type " but were not obtained from the pancreatic juice, instead representing the "Presssaft " of various organs. He notes that, *as a rule, the Presssaft of animal organs has a greater hydrolyzing action and less of a selective action than has pancreatic trypsin.* Abderhalden has fed polypeptides to dogs and rabbits or has injected them into the blood stream of dogs and rabbits, and notes that polypeptides which are not hydrolyzed by trypsin *in vitro* are burned in the animal body, the nitrogen being eliminated as urea. When, however, glycyl-glycine was injected subcutaneously into rabbits, it was eliminated in the urine as glycine, whereas when glycine alone was injected, it was burned in the organism. Even the racemic compounds appear to be split and completely burned when fed, but how the hydrolysis and utilization come about is still uncertain.

3. Polypeptides have been of value in detecting the presence of proteolytic enzymes. Polypeptides containing tyrosine, cystine, or tryptophane are particularly suited for this purpose. For example, glycyl-*l*-tyrosine is relatively soluble in water. The free tyrosine, however, is very insoluble. Similarly, the polypeptides containing cystine are usually relatively soluble. Cystine is almost insoluble. Polypeptides containing tryptophane do not give the characteristic rose-red color test of free tryptophane, when treated with dilute bromine water. When they are hydrolyzed, however, yielding free tryptophane, this very delicate color test can be used to indicate hydrolysis. Abderhalden has used rather extensively either the polypeptide, glycyl-*l*-tyrosine or " peptone roche "[8] for the identification of enzymatic action in tissues. If a section of an organ or tissue is covered with a solution of either glycyl-*l*-tyrosine or " peptone roche " and incubated at 37° for a time, tyrosine will crystallize out in stellate groups of needles upon those areas of the section where tryptic enzymes are present. Using this method, Abderhalden found that proteolytic enzymes first made their appearance in chick embryos which were at the seven or eight-day stage. Sections

[8] Abderhalden, E., and Steinbeck, E., Weitere Untersuchungen über die Verwendbarkeit des Seidenpeptons zum Nachweis peptolytischer Fermente, *Z. physiol. Chem.*, 68 : 312–316 (1910).

of 3.3 cm. pig embryos showed the presence of proteolytic enzymes in the liver and kidney areas. Sections of 3.2 cm. embryos, however, showed no evidences of the presence of proteolytic enzymes.

Many other similar studies have been conducted by various workers. Those who may be interested are referred to the files of the scientific journal, *Fermentforschung*.

The enzymatic decomposition of polypeptides may likewise be followed by placing the optically active polypeptide solution, together with the enzyme, in the tube of a polarimeter and noting changes in optical rotation. When this method is used, care must be taken to use comparison samples in which the enzyme alone and the polypeptide plus boiled enzyme are observed.

4. As already noted, polypeptides have been used to differentiate proteolytic enzymes of the tryptic and peptic types. Using polypeptides, Abderhalden found that whereas the pancreatic enzymes do not normally flow backward into the stomach, such flow may occur when the diet is abnormally rich in fat.

Within the tryptic group, polypeptides may differentiate between the various enzymes. Thus, for example, *d*-alanyl-*d*-alanine and *d*-alanyl-*l*-leucine are both rapidly hydrolyzed by the endotrypsin of yeast; they are much more slowly hydrolyzed by erepsin, and are practically unattacked by pure trypsin.

Long polypeptide chains may be differentially hydrolyzed at various linkages, depending upon the source of the tryptic enzyme which is employed. Polypeptides have been used to demonstrate the elaboration of proteolytic enzymes in the blood sera of animals injected with foreign protein. The normal blood sera of the horse or dog do not hydrolyze glycyl-*l*-tyrosine, although this polypeptide is hydrolyzed by the blood sera of the rabbit and the guinea pig. When, however, egg white or horse serum is injected into a dog and the dog allowed to become an anaphylactic reactor (*vide infra*), the blood serum acquires the property of hydrolyzing this polypeptide. Apparently the enzymes are elaborated in order to rid the blood stream of the foreign proteins, but the enzymes which are elaborated are not specific for the foreign protein. The elaboration of enzymes in the blood serum is not peculiar to the proteolytic enzymes. The intravenous injection of proteins will give rise to the formation of proteolytic enzymes. The intravenous injection of lactose or cane sugar will give rise to the elaboration of carbohydrases, and the injection of fat will give rise to the elaboration of lipases. Polypeptides, however, do provide a convenient method of detecting the presence of relatively small quantities of proteolytic enzymes.

CHAPTER XIII

THE ANALYSIS OF PROTEINS

Various methods have been proposed for the study of the amino-acid content of proteins and the identification of the various linkages or of the individual amino acids. By the use of certain of these methods we can prove either the presence or the absence of individual amino acids or the presence or absence of certain groups of amino acids. Some of the methods give only qualitative information, others give quantitative.

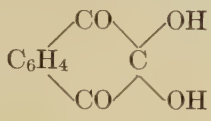
It would be beyond the scope of the present work to consider any of these methods in sufficient detail to provide a complete manual for the laboratory worker. The present discussion, therefore, will be limited to a consideration of the various technics which have been employed, with the literature citations so that the research worker may have the necessary information as to where the exact technic can be obtained and also some idea as to the limitations of the proposed method.

Color Tests.—Certain color reactions, characteristic of either amino acids or of linkages, have been proposed. These can be divided into two groups, those which are general for either linkages or for all amino acids, and those which are specific for some particular amino acid.

A. **General.**—1. *The Biuret Reaction.*—The biuret reaction is characteristic of the peptide (—CONH—) linkage. When a solution of protein or of polypeptides containing this linkage is treated with a dilute solution of copper sulfate, following which a dilute solution of sodium hydroxide is added, a characteristic blue-violet to violet-pink color reaction occurs. This test is given by all native proteins and most of their split products. The longer chains of proteins, in general, yield a blue-violet coloration, but as the chain becomes shorter, the color grades more and more toward the pink. The color is due to the formation of a substituted biuret, NHR—CO—NH—CO—NHR′, which reacts with the copper hydroxide and alkali to form a colored complex. Urea, when heated, yields biuret, NH_2—CO—NH—CO—NH_2. Histidine gives the biuret reaction. Therefore, the test is not specific for the peptide linkage. However, both biuret and histidine give colors characteristic of the pink end of the color series, and as additional radicals are added to

the biuret nucleus, the coloration becomes more and more a clear blue-violet. A blue-violet almost certainly indicates the presence of protein or a long polypeptide chain. A single peptide linkage, such as that present in glycyl-glycine, will not give the biuret reaction. Three or more amino acids must be linked together in order to show the biuret test. The test is of value in determining the presence or absence of small quantities of protein in biological fluids.

2. *The Ninhydrine Reaction.*—When protein split products or α-amino acids or any compound containing an α-amino group are treated with " ninhydrine " (triketo hydrindene hydrate),

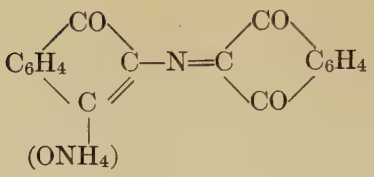


in aqueous solution, color is developed, the color ranging from a clear, deep blue to a violet-pink or even red. This reaction was first noted by Ruhemann,[1] and has been studied by Harding and MacLean[2] as a quantitative method for the determination of amino acids. At least one free $—NH_2$ group must be present in order that color may be developed. Ninhydrine is probably the most delicate reagent for detecting the presence of protein or of an α-amino acid. With some amino acids, one part in 100,000 parts of water can be detected.

Harding and MacLean note that the reaction is much more delicate if carried out in the presence of a small amount of pyridine, probably due to the buffer action of the pyridine. The ninhydrine reaction can be applied to the quantitative determination of an amino acid, providing that only a single amino acid is present in the solution. It is not, however, applicable to the quantitative estimation of amino acids when one is dealing with a mixture of various amino acids, due to the fact that the various amino acids give not only differences in the shade of color but differences in the depth of color per unit of the $—NH_2$ radical. Thus, using glycine as a standard, one can accurately determine quantitatively small amounts of glycine. The values, however, for alanine, aspartic acid, etc., determined by a glycine standard, will be erroneous. The color change which is developed is that of a substituted ammonium salt of diketo-hydrindylidene-diketo-hydrindamine,

[1] Ruhemann, S., Triketohydrindene Hydrate, *J. Chem. Soc.*, London, 97 : 2025–2031 (1910).

[2] Harding, V. J., and MacLean, R. M., A Colorimetric Method for the Estimation of Amino-Acid α-Nitrogen, *J. Biol. Chem.*, 20 : 217–230 (1915).



3. *Folin's Reagent.*—Folin[3] has proposed the use of β-naphthoquinone sulfonic acid as a general reagent for the colorimetric estimation of amino acids in blood. In the presence of strong alkalies and of an amino acid, this reagent develops a striking, deep red color. Ammonia likewise yields a color, but ammonia is readily removed, so that its interference would not be serious. Folin notes that urea, uric acid, creatinine, creatine, and hippuric acid do not yield colors. A color is developed with most of the nitrogen bases, such as the alkaloids or aniline, but as a rule these compounds are not present in solutions where one wishes to determine the presence or absence of amino acids.

B. **Specific Reactions.**—1. *The Millon Reaction.*—A red color is developed in many instances when a protein or a protein hydrolysate is heated with a solution of mercurous nitrate containing oxides of nitrogen or nitrous acid. *The red coloration is specific for the phenol group,* and whereas tyrosine is the only protein decomposition product which has been proven to contain the phenolic —OH, the reaction is, in general, considered as specific for tyrosine. The test is capable of great delicacy when properly carried out, *i.e.*, when a considerable excess of the reagent is avoided.

2. *The Xanthoproteic Reaction.*—When protein is treated with nitric acid, a yellow coloration is produced which is intensified to orange on the addition of ammonium hydroxide. The yellow coloration so commonly produced on skin which has come in contact with nitric acid is an example of the xanthoproteic reaction and indicates the presence of *nitrated protein.* Nitration takes place on the benzene rings, and the reaction is, therefore, specific for *aromatic nucleii* which are easily nitrated (tryptophane or tyrosine). The benzene nucleus of phenylalanine is not readily nitrated. Accordingly, proteins containing no other aromatic nucleii excepting those in phenylalanine do not show the xanthoproteic reaction.

3. *Liebermann's Reaction.*—Most proteins, when heated in solution with concentrated hydrochloric acid, yield a violet or blue-black coloration. The production of such a color is specific for *tryptophane or indole derivatives* (*vide infra*).

[3] Folin, O., A System of Blood Analysis. Supplement III. A New Colorimetric Method for the Determination of the Amino-Acid Nitrogen in Blood, *J. Biol. Chem.*, 51 : 377–391 (1922).

4. *Acree-Rosenheim Reaction.*—If a protein or a mixture of amino acids is treated with a small amount of formaldehyde and then warmed with concentrated hydrochloric acid, a violet to black coloration is produced. This is another modification of the Liebermann reaction and is specific for tryptophane or indole derivatives. This reaction is used to detect the presence of formaldehyde added to milk. The casein of milk contains tryptophane. The formaldehyde accentuates the delicacy of the tryptophane reaction.

5. *The Adamkiewicz Reaction.*—This is a further modification of the reactions specific for tryptophane or indole derivatives. A small amount of acetic acid is added to a protein solution or to a mixture of amino acids, and concentrated sulfuric acid is carefully layered underneath the solution. A violet to black ring at the interface between the sulfuric acid and the solution is indicative of tryptophane. The acetic acid contains glyoxylic acid, $COOH—CH—(OH)_2$, which breaks down to form an aldehyde, the aldehyde being the actual reagent reacting with the tryptophane in the presence of acid, to form the color.

6. *The Benzaldehyde Reaction.*—Benzaldehyde, or preferably *p*-dimethyl-amino-benzaldehyde (Ehrlich's reagent), is specific for the indole nucleus and, therefore, for tryptophane. When a protein or a mixture of amino acids containing tryptophane is treated with benzaldehyde or Ehrlich's reagent in the presence of 20 per cent hydrochloric acid, an intense, clear blue coloration results. This again is merely another modification of the tryptophane-aldehyde reactions noted under Nos. 3, 4, and 5, above. The test is often employed to detect the presence of indole-forming bacteria.

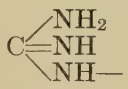
7. *Reduced Sulfur Test.*—In many instances a black coloration is produced when a protein or a mixture of amino acids is heated with alkali and lead acetate. The coloration is due to the formation of black lead sulfide and appears to be specific for the presence of cystine or cysteine, or rather the groups, —S—S— or —SH. Whether or not there are other sulfur linkages in proteins which will yield this reaction is still uncertain.

8. *The Molisch Test.*—The Molisch test is specific for carbohydrates. Many proteins contain a carbohydrate radical. In testing for such a radical, a trace of α-naphthol is added to a protein solution and this is layered over concentrated sulfuric acid. A pink to red coloration at the interface indicates the presence of carbohydrate. This test is probably dependent upon the formation of furfural from the carbohydrate.

9. *The Sakaguchi Test.*—Sakaguchi[4-5] has proposed a color reaction

[4] Sakaguchi, S., Über eine neue Farbenreaktion von Protein und Arginin, *J. Biochem.*, Tokyo, 5 : 25–31 (1925).

which he claims to be specific for the *free-guanidine* group, and, therefore, for arginine. To 5 cc. of a 1 per cent solution of a protein, or of a 1 per cent solution of an amino-acid mixture containing arginine, is added 2 cc. of a 15 per cent solution of sodium hydroxide, followed by 5 cc. of a 0.15 per cent solution of α-naphthol, and the entire mixture treated with 0.3 N sodium hypochlorite. After standing at 2° to 4° C. for forty minutes, the mixture is diluted and read against a standard solution in a colorimeter. The color which is developed is an intense red. Only compounds containing the free-guanidine group,



react. Accordingly the color test should be specific for arginine. In later papers Sakaguchi[6,7] shows that the guanidine group of arginine is free in most native proteins and also that the proteins differ widely in the rate with which arginine is set free when the proteins are hydrolyzed by enzymes or by acid or alkali methods of hydrolysis.

The Quantitative Determination of Amino and Carboxyl Groups in Amino Acids or Mixtures of Amino Acids.—A. *Van Slyke's Method.*—Van Slyke[8,9] has devised an apparatus whereby one can determine quantitatively with a high degree of accuracy the primary amino nitrogen which is present in a protein, an amino acid, or a mixture of amino acids. Van Slyke makes use of the reaction between an aliphatic amino group and nitrous acid,

$$R—NH_2 + HNO_2 = R—OH + N_2 + H_2O,$$

the nitrogen gas evolved by the reaction being collected and measured. Van Slyke gives tables for the conversion of the volume of the nitrogen gas into milligrams of amino nitrogen. This method has proven of very great service not only in a study of the proteins but in the rapid analysis of amino acids and in the determination of relatively small amounts of amino acids in biological fluids. However, the ϵ group of lysine reacts

[5] Sakaguchi, S., Über die Bindungsweise und quantitative Bestimmung des Arginins im Proteinmoleküle, *J. Biochem.*, Tokyo, 5 : 133–142 (1925).

[6] Sakaguchi, S., Über Dearginoprotein, *J. Biochem.*, Tokyo, 5 : 143–157 (1925).

[7] Sakaguchi, S., Über die Spaltung des Proteinkörpers durch Alkali, *J. Biochem.*, Tokyo, 5 : 159–169 (1925).

[8] Van Slyke, D. D., A Method for Quantitative Determination of Aliphatic Amino Groups. Applications to the Study of Proteolysis and Proteolytic Products, *J. Biol. Chem.*, 9 : 185–204 (1911).

[9] Van Slyke, D. D., The Quantitative Determination of Aliphatic Amino Groups. II. *J. Biol. Chem.*, 12 : 275–284 (1912).

somewhat more slowly than does the α-amino group. Only the primary amino group reacts.

This method of Van Slyke for the determination of free amino nitrogen has been of particular value in a variety of problems.

With the micro-apparatus one can quantitatively determine 0.0005 grams or less of amino nitrogen. Thus, only extremely small quantities of an amino acid are necessary in order to determine the amino nitrogen content and accordingly its purity.

The rate or extent of protein hydrolysis can be accurately followed by determining the free amino nitrogen at various intervals of time, since when the peptide linkage is broken, a free amino group is formed.

Similarly, the relative digestibility of proteins *in vitro*, *i.e.*, the rate of hydrolysis by a particular enzyme or by a mixture of enzymes can be followed in a quantitative manner.

The method affords information as to whether or not a given mixture contains proteolytic enzymes. If there is a progressive increase in free amino nitrogen, it can be taken as evidence that enzymatic action is present.

Van Slyke has shown that the ϵ —NH_2 group of lysine exists free in native proteins and that the free —NH_2 nitrogen content of native proteins can be correlated with their lysine content. There is still some doubt as to whether this is invariably the case. It appears, however, to afford at least a close approximation of the lysine content.

B. *The Sörensen Titration.*—Sörensen[10] proposed a method whereby the carboxyl group of an amino acid could be titrated directly in aqueous solution with a standard alkali. Amino acids dissolve in water to form an essentially neutral solution, due to their amphoteric nature, the acidity of the carboxyl group being masked by the basicity of the amino group. Sörensen added formaldehyde to the solution of amino acids, resulting in the formation of either a methylene derivative or of an aldehyde ammonia.

$$R—CH(NH_2)COOH + HCHO = R—CH(N{=}CH_2)COOH + H_2O$$

or

$$R—CH(NH_2)COOH + HCHO = R—CH(NH—CH_2OH)COOH$$

In this way the basicity of the amino group is masked, and it is possible to titrate the carboxyl group with standard sodium hydroxide, using phenolphthalein as an indicator.

Sörensen's titration method is of value not only in quantitatively determining the carboxyl groups in an amino acid or a mixture of amino

[10] Sörensen, S. P. L., Enzymstudien, *Biochem. Z.*, 7 : 45–101 (1907).

acids, but likewise in quantitatively determining amino groups in the presence of other organic acids. In this case, this mixture of amino acids and organic acids is titrated in aqueous solution with standard sodium hydroxide, using phenolphthalein as an indicator, until the organic acids which are present are completely neutralized. Neutral formaldehyde is then added to mask the amino groups of the amino acids which are present, and the solution can be further titrated with standard sodium hydroxide. Sörensen's method has proven of great value in enzyme studies, inasmuch as it permits one to follow the rate at which the protein is being hydrolyzed, since the opening of the peptide linkage results in the formation of additional carboxyl groups.

C. *Foreman's Titration.*—Foreman[11] noted that when amino acids or even ammonium salts are titrated in 85 per cent ethyl alcohol, the ammonium radical or the amino groups do not react basic. The acid groups with which the ammonium radical is associated, or the carboxyl group of the amino acids can accordingly be titrated directly in 85 per cent alcohol, using phenolphthalein as an indicator. Thus, for example, a 0.1 N solution of ammonium chloride titrates in 85 per cent alcohol as though it were a 0.1 N solution of hydrochloric acid. It is sometimes more convenient to use Foreman's titration method than it is to use Sörensen's method. Martens[12] has made a careful comparative study of the Van Slyke, Sörensen, and Foreman methods. He finds that using phenolphthalein as an indicator, Foreman's method yields somewhat low results, Sörensen's method somewhat high results. He suggests a modification of Foreman's method, titrating the amino acid in 93 to 95 per cent alcohol and using thymolphthalein as an indicator. Under such conditions, the carboxyl groups can be determined with essentially the same degree of accuracy as is possible for the determination of the amino groups, using the Van Slyke method.

D. *Widmark and Larsson's Titration.*—Widmark and Larsson[13] suggest the estimation of amino acids by following the electrical conductivity of the amino-acid solution during the time that successive increments of sodium hydroxide are added to the solution. They used a dipping conductivity cell and plotted the conductivity values for the readings of the Wheatstone bridge, $\frac{a}{1000 - a}$, against the volume of

[11] Foreman, F. W., Rapid Volumetric Methods for the Estimation of Amino-Acids, Organic Acids and Organic Bases, *Biochem. J.*, 14 : 451–473 (1920).

[12] Martens, R., Considérations sur le dosage séparé des acides aminés et des polypetides dans les produits de digestion des protéines, *Bull. Soc. chim. biol.*, 9 : 454–482 (1927).

[13] Widmark, E. M., P., and Larsson, E. L., Bestimmung von Aminosäuren mittels konduktometrischer Titration, *Biochem. Z.*, 140 : 284–294 (1923).

standard sodium hydroxide which was added. They noted that there was a change in slope of the conductivity curve at the point where the carboxyl group of the amino acid was exactly neutralized. They present curves for the conductiometric titration of glycine, alanine, isoserine, α-amino valerianic acid, leucine, norleucine, tryptophane, tyrosine, aspartic and glutamic acids, arginine, histidine hydrochloride, and lysine hydrochloride. The carboxyl group of arginine and histidine showed no evidence of combination with sodium hydroxide. Evidently the remainder of the molecule was so basic as to inhibit salt formation. The conductiometric titration of lysine did not yield a sharp end-point. Figure 102 shows typical conductiometric titration curves as obtained for glycine (one carboxyl group), aspartic acid (two carboxyl groups), and tyro-

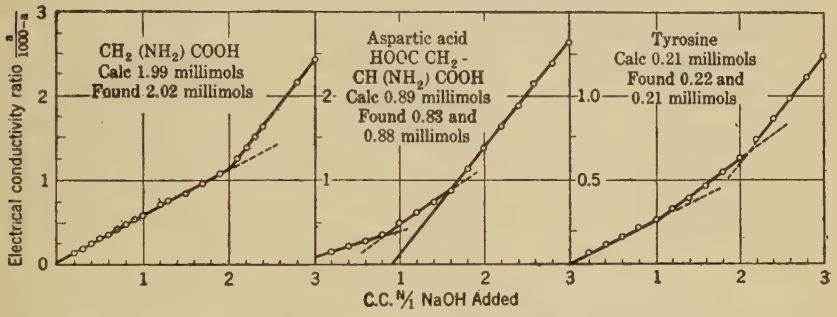


FIG. 102.—Conductiometric titration curves of amino acids with sodium hydroxide. (Data of Widmark and Larsson.)

sine (both the carboxyl and phenolic group reacting with the sodium hydroxide).

THE HYDROLYSIS OF PROTEINS.—For the quantitative determination of individual amino acids, it is necessary to hydrolyze the protein into its constituent amino acids. Various methods have been proposed, *i.e.*, hydrolysis by acids, hydrolysis by alkalies, and hydrolysis by enzymes. Each has its advantages and disadvantages.

A. *Hydrolysis by Acids.*—Constant-boiling (1.115 sp. g.) or approximately 20 per cent hydrochloric acid is most commonly used, although in some instances, it is advantageous to use 25 per cent (by weight) sulfuric acid. Hydrolysis by 25 per cent sulfuric acid is usually somewhat slower than hydrolysis with constant-boiling hydrochloric acid but reaches the same completeness of hydrolysis.

Proteins differ widely in the rapidity with which hydrolysis reaches completion. In some instances, a protein may be completely hydrolyzed by boiling for 6 to 8 hours with 20 per cent hydrochloric acid. In other instances, as much as twenty-four hours' boiling may be required.

Various other acids have been tested. Hydrofluoric acid is unsuited for protein work, the dilute acid reacting too slowly, whereas the concentrated acid yields not simple amino acids but complex polypeptides or condensation products.

Formic acid causes only partial hydrolysis. Certain amino acids are hydrolyzed off, but apparently an equilibrium is reached fairly early in the process. In addition, there is the danger of forming formic derivatives with the amino group. The remaining organic acids are too weak to produce complete hydrolysis. Recently Fodor and Epstein[14] have studied the degradation of gelatin in the presence of acetic anhydride. In some instances, amino acids or amino-acid derivatives were found to be acetylated. A considerable fraction of the products isolated was in the form of polypeptides, indicating that even acetic anhydride will not completely hydrolyze proteins.

B. *Hydrolysis by Alkalies.*—The alkalies, sodium hydroxide, potassium hydroxide, or barium hydroxide, cause very rapid and complete hydrolysis. Unfortunately alkaline hydrolysis results in the racemization of the amino acids, so that they are isolated to a large extent in the optically inactive form. Because of this, alkaline hydrolysis is rarely used. In addition, the strong alkalies bring about the decomposition, especially the deamination, of certain of the amino acids.

C. *Enzymatic Hydrolysis.*—As noted earlier, pepsin hydrolyzes proteins only to proteoses, peptones, and polypeptides. Trypsin, in general, hydrolyzes proteins to the constituent amino acids, but the process is slow and, in many instances, is not entirely complete. Accordingly, while tryptic hydrolysis is utilized in certain instances, it is not generally employed.

The Quantitative Study of the Hydrolytic Products of Proteins.—The methods for the quantitative study of the mixture obtained by the hydrolysis of proteins may be divided into two general groups, (A) those methods which are concerned with the identification of *groups of constituents*, and (B) those methods which are concerned with either the isolation or the quantitative estimation of *individual amino acids.*

A. Group Analysis.—1. *Hausmann's Method.*—Hausmann[15,16] proposed the characterization of a protein by distributing the nitrogen content of its hydrolytic decomposition products into three groups, *i.e.*, ammonia nitrogen, basic nitrogen, and non-basic nitrogen.

[14] Fodor, A., and Epstein, C., Über den Abbau der Gelatine durch Essigsäureanhydrid, *Z. physiol. Chem.*, 171 : 222–241 (1927).

[15] Hausmann, W., Ueber die Vertheilung des Stickstoffs im Eiweissmolekül, *Z. physiol. Chem.*, 27 : 95–108 (1899).

[16] Hausmann, W., Ueber die Vertheilung des Stickstoffs im Eiweissmolekül, II. *Z. physiol. Chem.*, 29 : 136–145 (1900).

Osborne[17] modified the Hausmann method by adding a fourth fraction, *i.e.*, that of humin nitrogen (older terminology = melanin nitrogen).

The Ammonia Nitrogen.—Following acid hydrolysis, the excess of acid is removed by distillation, the solution containing the hydrolysis products is then rendered alkaline, using either magnesium oxide or preferably a suspension of calcium hydroxide, and the ammonia distilled into standard acid, preferably under a partial vacuum. The ammonia which is formed has been generally regarded as being derived from acid amide linkages, —$CONH_2$, and in many instances, is referred to as the "amide nitrogen." Gortner and Holm[18] have shown that all of the amide nitrogen in a twenty-four to forty-eight hour hydrolysis is not derived from acid amide linkages but that there may be a very appreciable amount of deamination of the amino acids. They find a constant value for amide nitrogen during the first four hours of hydrolysis, following which deamination begins, and that there is an increase in ammonia nitrogen which continues and does not reach a constant value even at the end of a six week hydrolysis. Thus, with a 3-gram sample of fibrin, a one-hour hydrolysis yielded 34.50 milligrams of ammonia nitrogen; a four-hour hydrolysis, 34.70; a twelve-hour hydrolysis, 37.55; a twenty-four-hour hydrolysis, 40.65; a seventy-two-hour hydrolysis, 51.00; a two hundred and one-hour hydrolysis, 69.32; and a six-week hydrolysis, 100.30 milligrams.

From a 10-gram sample hydrolyzed for twenty-four hours, they obtained 137 milligrams of ammonia nitrogen. The ammonia-free hydrolysate was concentrated, re-acidified with hydrochloric acid to 20 per cent hydrochloric acid, and further boiling for thirteen days yielded an additional 115 milligrams of ammonia nitrogen. The ammonia-free solution from this determination was further boiled with 20 per cent hydrochloric acid for an additional six-week period, and a further 79.00 milligrams of ammonia nitrogen was obtained. An analysis of the chloroplatinates from these three fractions showed that each fraction was composed entirely of ammonia. Accordingly, there seems to be no doubt but that a certain amount of deamination does occur under the usual conditions of acid hydrolysis and that the true amide nitrogen value can only be ascertained by hydrolyzing for only relatively short periods of time. Gortner and Holm suggest that under the usual conditions of hydrolysis approximately 20 per cent of amide nitrogen of a

[17] Osborne, T. B., and Harris, I. F., Nitrogen in Protein Bodies, *J. Am. Chem. Soc.*, 25 : 323–353 (1903).

[18] Gortner, R. A., and Holm, G. E., The Effect of Prolonged Acid Hydrolysis upon the Nitrogen Distribution of Fibrin with Especial Reference to the Ammonia Fraction, *J. Am. Chem. Soc.*, 39 : 2736–2745 (1917).

twenty-four-hour hydrolysate may be due to deamination. They show, however, that the deamination affects only the monoamino acids; the basic amino acids, arginine, histidine, and lysine, are not deaminized even by boiling for six weeks with constant-boiling hydrochloric acid.

The Humin Nitrogen.—The humin (melanin) of a protein hydrolysate is the black, amorphous material which separates when proteins are hydrolyzed with strong acids. Gortner and coworkers[19-26] have shown that humin is formed by the condensation of tryptophane with an aldehyde, resulting in the production of the humin. The reaction apparently is with the indole nucleus, the condensation presumably taking place on the α-hydrogen. The colors produced in the Liebermann, Acree-Rosenheim, and Adamkiewicz reactions are due to the early stages of humin formation. The evidence presented by Gortner and his coworkers shows clearly that proteins contain either an aldehyde group or some other group which reacts as an aldehyde in condensing with tryptophane, resulting in the formation of humin. The nature of the compound to which this reactive group is attached, however, remains still to be determined.

Gortner and Holm suggest that the humin nitrogen determination can be so conducted as to be a quantitative measure of the tryptophane

[19] Gortner, R. A., and Blish, M. J., On the Origin of the Humin Formed by the Acid Hydrolysis of Proteins, *J. Am. Chem. Soc.*, 37 : 1630–1636 (1915).

[20] Gortner, R. A., The Origin of the Humin Formed by the Acid Hydrolysis of Proteins. II. Hydrolysis in the Presence of Carbohydrates and of Aldehydes, *J. Biol. Chem.*, 26 : 177–204 (1916).

[21] Gortner, R. A., and Holm, G. E., On the Origin of the Humin Formed by the Acid Hydrolysis of Proteins. III. Hydrolysis in the Presence of Aldehydes. II. Hydrolysis in the Presence of Formaldehyde, *J. Am. Chem. Soc.*, 39 : 2477–2501 (1917).

[22] Holm, G. E., and Gortner, R. A., On the Origin of the Humin Formed by the Acid Hydrolysis of Proteins. IV. Hydrolysis in the Presence of Aldehydes. III. Comparative Hydrolysis of Fibrin and Gelatin in the Presence of Various Aldehydes, *J. Am. Chem. Soc.*, 42 : 632–640 (1920).

[23] Gortner, R. A., and Holm, G. E., The Origin of the Humin Formed by the Acid Hydrolysis of Proteins. V., *J. Am. Chem. Soc.*, 42 : 821–827 (1920).

[24] Holm, G. E., and Gortner, R. A., The Humin Formed by the Acid Hydrolysis of Proteins. VI. The Effect of Acid Hydrolysis Upon Tryptophane, *J. Am. Chem. Soc.*, 42 : 2378–2385 (1920).

[25] Gortner, R. A., and Norris, E. R., The Origin of the Humin Formed by the Acid Hydrolysis of Proteins. VII. Hydrolysis in the Presence of Ketones, *J. Am. Chem. Soc.*, 45 : 550–553 (1923).

[26] Burr, G. O., and Gortner, R. A., The Humin Formed by the Acid Hydrolysis of Proteins. VIII. The Condensation of Indole Derivatives with Aldehydes, *J. Am. Chem. Soc.*, 46 : 1224–1246 (1924).

content of a protein. They found that when fibrin was hydrolyzed in the presence of increasing amounts of formaldehyde, the black, acid-insoluble humin was increased from 9.60 milligrams per 3 grams of fibrin, where no formaldehyde was added, to a maximum of 19.02 milligrams of acid-insoluble humin nitrogen, where 0.10 gram of formaldehyde in the form of trioxymethylene was added. The addition of larger amounts of trioxymethylene sharply decreased the amount of acid-insoluble humin nitrogen. They believe the 19.02 milligrams of acid-insoluble humin nitrogen to be a practically quantitative measure of the tryptophane nitrogen which was present in the original 3 grams of fibrin.

The humin nitrogen, as obtained in an ordinary protein hydrolysate, bears little or no relationship to the true tryptophane nitrogen content of the protein, inasmuch as the humin formation depends upon two variables, the tryptophane content and the presence of an adequate amount of aldehyde. In all the proteins studied by Gortner and his coworkers, the tryptophane and aldehyde components were not in the proper ratio for the quantitative conversion of tryptophane into humin. Accordingly, the humin formed in a protein hydrolysis as ordinarily carried out, while dependent upon the tryptophane content of the protein, is not a quantitative measure of that tryptophane content.

The Basic Nitrogen.—In the Hausmann method the basic nitrogen is determined by precipitating the diamino acids, arginine, histidine, lysine, and cystine, with phosphotungstic acid in the presence of an excess of hydrochloric acid. The phosphotungstates of these amino acids are nearly insoluble. The phosphotungstate precipitate is filtered off, washed with a dilute solution of phosphotungstic acid in dilute hydrochloric acid, and the nitrogen in the entire precipitate determined by the Kjeldahl method.

The Non-Basic Nitrogen.—The non-basic nitrogen is determined by kjeldahling an aliquot of the filtrate from the phosphotungstate precipitate. This fraction of nitrogen represents the monoamino monocarboxylic acids and the monoamino dicarboxylic acids.

Thus, the Hausmann method divides the nitrogen of a protein hydrolysate into four fractions. The *advantages* of the method are that it is rapid, that it requires only a small amount of protein, 0.5 grams to 1 gram, and that by use of this method one can obtain rather definite information as to the ratio existing between the diamino acids and the monoamino acids comprising the protein molecule. The *disadvantages* of the method are that the method gives no clue as to the presence or absence of any individual amino acid with the possible exception of tryptophane, and that there are many proteins having somewhat similar con-

tent of basic and non-basic amino acids, such proteins being more or less indistinguishable from each other by the Hausmann technic.

2. *Van Slyke's Method.*—Van Slyke[27-29] has made use of his method for determining amino nitrogen and the ratio between total nitrogen and free-amino nitrogen in certain of the amino acids, together with the Hausmann procedure, in order to estimate quantitatively certain of the amino acids in proteins. In Van Slyke's method the protein is hydrolyzed, and the *acid amide nitrogen* and *humin nitrogen* determined essentially as in Hausmann's method.

The *diamino acids* are precipitated with phosphotungstic acid in 5 per cent (by weight) hydrochloric acid, and the basic phosphotungstates are filtered off and washed with dilute phosphotungstic acid in dilute hydrochloric acid. The basic phosphotungstate precipitate is then dissolved in very dilute alkali and decomposed by the addition of 20 per cent barium chloride solution, the insoluble barium phosphotungstate is filtered off and washed, and the filtrate containing the basic amino acids is slightly acidified and concentrated to a definite volume. This filtrate contains the arginine, histidine, lysine, and a part of the cystine which were present in the original protein. These four amino acids are then estimated as follows:

a. Total sulfur is determined on an aliquot of this solution as barium sulfate by the method of Benedict,[30] as modified by Denis,[31] and from the weight of barium sulfate obtained, the *cystine nitrogen* content is calculated.

b. The total nitrogen present in the basic fraction is determined on an aliquot of the solution of the bases.

c. The free-amino nitrogen content is determined by Van Slyke's method in an aliquot of the solution.

d. Sufficient potassium hydroxide is added to an aliquot of the bases to make a solution containing 50 per cent of potassium hydroxide. This

[27] Van Slyke, D. D., The Analysis of Proteins by Determination of the Chemical Groups Characteristic of the Different Amino-Acids, *J. Biol. Chem.*, 10 : 15–55 (1911).

[28] Van Slyke, D. D., Improvements in the Method for Analysis of Proteins by Determination of the Chemical Groups Characteristic of the Different Amino-Acids, *J. Biol. Chem.*, 22 : 281–285 (1915).

[29] Van Slyke, D. D., Analysis of Proteins by Determination of the Chemical Groups Characteristic of the Different Amino-Acids. Correction, *J. Biol. Chem.*, 23 : 411 (1915).

[30] Bendict, S. R., The Estimation of Total Sulphur in Urine, *J. Biol. Chem.*, 6 : 363–371 (1909).

[31] Denis, W., The Determination of Total Sulphur in Urine, *J. Biol. Chem.*, 8 : 401–403 (1910).

solution is boiled for six hours under such conditions that the volume remains constant and that any ammonia which is evolved is collected in standard acid. By this process arginine is decomposed into urea and ornithine, the urea being further broken up into ammonia and carbon dioxide.

$$\begin{matrix} & NH_2 \\ & / \\ C & = NH \\ & \backslash \\ & NH—(CH_2)_3—CH(NH_2)COOH \end{matrix} + KOH =$$

$$\underset{\text{arginine}}{NH_2—CO—NH_2} + \underset{\text{urea}}{NH_2—(CH_2)_3—CH(NH_2)COOH}$$

$$\underset{\text{ornithine}}{}$$

$$NH_2—CO—NH_2 \rightarrow 2\ NH_3 + CO_2$$

Consequently half of the arginine nitrogen has been evolved as ammonia. From the amount of nitrogen which is evolved as ammonia, the quantity of *arginine nitrogen* in the original solution is calculated.

e. We now have values for cystine, arginine, total nitrogen, and amino nitrogen. The amount of *histidine* which is present is accordingly secured by a mathematical calculation, inasmuch as histidine and arginine are the only basic amino acids which contain non-amino nitrogen. Two-thirds of the histidine nitrogen and three-fourths of the arginine nitrogen are non-amino nitrogen (the $—NH_2$ group of the guanidine nucleus does not react with nitrous acid). Accordingly the non-amino nitrogen of the bases minus three-fourths of the arginine nitrogen is equal to two-thirds of the histidine nitrogen, which permits us to calculate the amount of *histidine nitrogen* in the basic fraction.

f. The *lysine nitrogen* is finally obtained by subtracting from the total nitrogen, the sum of the arginine nitrogen plus the histidine nitrogen plus the cystine nitrogen.

Van Slyke further differentiates the nitrogen in the filtrate from the bases by use of his amino nitrogen apparatus into (1) amino nitrogen, and (2) non-amino nitrogen, the former being derived from the monoamino monocarboxylic and monoamino dicarboxylic acids, the latter being derived from proline, oxyproline, and in part from tryptophane.

Van Slyke's method has been used very extensively in protein studies. Larmour[32] has collected the various analyses which have been published, in order to subject them to statistical analyses. We are not at this point concerned with his statistical findings. His paper, however, can con-

[32] Larmour, R. K., Correlations Between the Total Nitrogen of the Bases and Arginine Nitrogen and between Total Nitrogen of the Bases and Lysine Nitrogen, of Various Proteins, *Trans. Roy. Soc.*, Canada, Section V, 349–363 (1923).

veniently be used as a source of reference for an extensive series of protein analyses.

Van Slyke's method has the *advantages* of requiring a relatively small amount of protein, 3 grams or less, and of permitting the more or less quantitative determination of arginine, histidine, and lysine. The cystine value which is found by Van Slyke's method is too low, inasmuch as cystine is partially decomposed when boiled with acids, the portion which is not decomposed being either racemized or converted into an isomeric form, the phosphotungstate of which is appreciably soluble (cf. Hoffman and Gortner).[33]

Gortner and Sandstrom[34] have made a critical study of Van Slyke's method, using mixtures of known amino acids in the study. They reach the conclusion that the histidine and lysine values may be somewhat in error when tryptophane and proline are present. The arginine which was added was determined within experimental error. The cystine nitrogen was in three experiments 64.5, 62.1, and 73.3 per cent of the cystine nitrogen added. The histidine values were satisfactory in the absence of tryptophane or proline but were high in the presence of either one or both of these amino acids. The lysine values were only slightly high and might well have been attributed to experimental errors.

The *disadvantages* of Van Slyke's method lie in the fact that it gives us no insight into the composition of the group of monoamino monocarboxylic and monoamino dicarboxylic acids.

B. Isolation and Identification of Individual Amino Acids.—1. *Fischer's Ester Method.*—In Fischer's study of amino-acid derivatives he observed that the ethyl esters of the monoamino monocarboxylic and monoamino dicarboxylic acids could be distilled *in vacuo* without appreciable decomposition. It is upon this observation that he based his ester method.

The protein is hydrolyzed by hydrochloric acid; the excess of hydrochloric acid is removed by distillation; the mixture of amino acid hydrochlorides is concentrated; and the concentrated solution is saturated with gaseous hydrochloric acid. On standing at a low temperature, *glutamic acid hydrochloride* crystallizes out. This is filtered off and recrystallized from concentrated hydrochloric acid.

The remaining amino acids are then converted into their ethyl esters by boiling with absolute ethyl alcohol in the presence of hydrochloric

[33] Hoffman, W. F., and Gortner, R. A., Sulfur in Proteins. I. The Effect of Acid Hydrolysis upon Cystine, *J. Am. Chem. Soc.*, 44 : 341–360 (1922).

[34] Gortner, R. A., and Sandstrom, W. M., Proline and Tryptophane as Factors Influencing the Accuracy of Van Slyke's Method for the Determination of Nitrogen Distribution in Proteins, *J. Am. Chem. Soc.*, 47 : 1663–1671 (1925).

acid or zinc chloride. On concentrating and cooling the mixture of esters, *glycine ester hydrochloride* crystallizes out and can be removed by filtration. The excess of hydrochloric acid in the remaining mixture of esters is then neutralized by some appropriate technic. The preferable method is to accurately determine the amount of hydrochloric acid which is present and add an exact equivalent of sodium ethylate dissolved in absolute alcohol. The free esters are now soluble in absolute ether. The ethereal solution is dried and submitted to vacuum distillation, the following fractions being secured,

60° (10 mm.) = glycine, alanine, leucine, proline.
60°–100° (10 mm.) = valine, leucine, proline.
100°–130° (0. 5 mm.) = leucine and proline.
130°–180° (0. 5 mm.) = phenylalanine, glutamic acid, aspartic acid, and serine.

It will be noted that there is a considerable overlapping of amino acids in the various fractions. The residue which does not distill contains arginine, histidine, lysine, tyrosine, cystine, oxyproline, and various anhydrides, such as leucine imide, etc., and other diketopiperazines formed by secondary reactions.

The ester fractions, as obtained in the vacuum distillation, are hydrolyzed by boiling with water, and the individual amino acids separated by a process of fractional crystallization.

It is obvious that such a method is far from quantitative. Osborne and Jones,[35] after many years of experience in working with Fischer's ester method, undertook a study of the method, in order to ascertain the errors which were involved. They list the following sources of error, (1) incomplete hydrolysis, (2) loss of amino acids, due to the formation of humin, (3) incomplete esterification, (4) the decomposition of the esters by hydrolysis prior to distillation, (5) unavoidable loss in separating the amino acids by fractional crystallization. To the above, we must undoubtedly add (6) the conversion of amino acid esters into diketopiperazine derivatives. In this experiment, they mixed 326 grams of pure amino acids (omitting the boiling with hydrochloric acid for twenty-four hours), immediately proceeded to the esterification of this mixture of amino acids, to the liberation of the free esters and to distillation *in vacuo*. The various fractions were then worked up for their amino-acid content. Table XLVII shows the grams of amino acids which they submitted to esterification, the yield of amino acids which were recovered, and the percentage recovery. The distillable esters, as obtained, were

[35] Osborne, T. B., and Jones, D. Breese, A Consideration of the Sources of Loss in Analyzing the Products of Protein Hydrolysis, *Am. J. Physiol.*, 26 : 305–328 (1910).

only 73 per cent of the theoretical amount, and the total recovery was only 66.17 per cent of the theory. This probably explains why most of the analyses of proteins by Fischer's ester method range from 40 to 60 per cent of the weight of the protein taken.

It should be pointed out here that the weight of amino acids theoretically obtainable, exceeds considerably the weight of the protein from which the amino acids are derived, inasmuch as the elements of water are added to each peptide linkage. A protein should yield from 110 to 120 per cent of its weight of amino acids. The isolation of 60 per cent of the amino acids in a protein analysis means, therefore, that approximately only half of the amino acids which are actually present have been accounted for.

TABLE XLVII

Showing the Recovery of Amino Acids from a Known Mixture by Fischer's Ester Method (Data of Osborne and Jones)

Amino Acids Taken		Amino Acids Recovered	
	Grams	Grams	Per Cent
Alanine	16.00	7.34	45.88
Valine	4.00	1.64	41.00
Leucine	85.00	69.36	81.60
Proline	31.00	22.56	72.77
Phenylalanine	26.00	18.07	69.51
Aspartic acid	6.00	2.55	42.50
Glutaminic acid	120.20	83.26	69.39
Tyrosine	16.00	7.97	49.81
Arginine	5.50	3.57	64.91
Histidine	2.04	0.80	39.21
Serine	2.00	0.00	0.00
Ammonia	14.31		
Total	328.05	217.12	66.17

2. *Dakin's Method.*—Dakin [36,37] has introduced a new method which also has advantages either in replacing Fischer's method or in supplementing it. In Dakin's method, the protein is hydrolyzed with sulfuric acid; the sulfuric acid is quantitatively removed with barium hydroxide; and the aqueous solution of amino acids concentrated until crystallization of amino acids begins. This thick, semi-crystalline mass

[36] Dakin, H. D., On Amino-Acids, *Biochem. J.*, 12 : 290–317 (1918).

[37] Dakin, H. D., Amino-Acids of Gelatin, *J. Biol. Chem.*, 44 : 499–529 (1920).

is then extracted in a continuous liquid extractor with normal butyl alcohol. The amino acids themselves, with the exception of proline, are insoluble in anhydrous normal butyl alcohol, but the monoamino monocarboxylic acids are slightly soluble in normal butyl alcohol saturated with water. The aqueous butyl alcohol distills at a lower temperature than does absolute butyl alcohol. Accordingly there is left in the receiving flask a crystalline mass of the monoamino monocarboxylic acids. The absolute butyl alcohol containing the proline is removed from this crystalline mass, and the crystalline mass is subjected to fractional crystallization for its individual constituents, or it may be esterified and the esters distilled as in Fischer's method.

The solution which has been extracted with butyl alcohol contains the diamino acids, the dicarboxylic acids, and tyrosine. The basic amino acids can be removed by precipitation with phosphotungstic acid. Tyrosine, because of its insolubility in water can be readily obtained, and the dicarboxylic acids are then separated by fractional crystallization.

It was by use of this modification that Dakin found in excess of 10 per cent of a new amino acid, β-hydroxyglutamic acid in casein.

Dakin's method of extraction with butyl alcohol affords in many instances a valuable procedure for the purification of an individual amino acid. Amino acids which cannot be readily obtained in a crystalline form from aqueous solution, readily crystallize when extracted from the aqueous solution with normal butyl alcohol in a continuous extractor.

Fischer's method and Dakin's method have the great *advantage* that they yield the individual acids in a pure crystalline condition. Accordingly, one or another modification of these methods must be used, if one wishes to prepare quantities of the crystalline amino acids. Their *disadvantage* lies in the fact that neither method affords a quantitative separation and estimation of any particular amino acid, and the large amounts of protein which are required (100 to 500 grams) are so great as to preclude the use of these methods in many instances. Similarly the methods are roughly quantitative only when carried out by the most expert technicians.

3. *Kossel's Separation of the Bases.*—Kossel and Kutscher[38] suggested a method whereby the diamino acids, arginine, histidine, and lysine, could be prepared in pure form and quantitatively determined with a fairly high degree of precision.

The protein is hydrolyzed with sulfuric acid, and the sulfuric acid later removed quantitatively with barium hydroxide. A hot, saturated solution of silver sulfate is then added to the aqueous solution of amino

[38] Kossel, A., and Kutscher, F., Beiträge zur Kenntniss der Eiweisskörper, *Z. physiol. Chem.*, 31 : 165–214 (1900).

acids until a drop of the silver amino-acid solution turns to a brown color on the addition of barium hydroxide, indicating the formation of silver oxide and accordingly an excess of silver in the solution. The solution is then saturated with powdered barium hydroxide, precipitating the silver salts of *arginine* and *histidine*. *Lysine* remains in solution and can be precipitated later by phosphotungstic acid.

The precipitate containing the silver salts of arginine and histidine is filtered off, the silver removed with hydrogen sulfide, and the solution of amino acids filtered from the resulting precipitate of silver sulfide. The excess of hydrogen sulfide is removed by aeration. Hot, concentrated silver sulfate solution is again added until an excess of silver is present. At this point barium hydroxide solution is added until the solution is neutral. Histidine silver precipitates at neutrality, leaving the arginine silver in solution. The histidine silver is filtered off, decomposed with hydrogen sulfide, and the solution of the free amino acid concentrated to a small volume. The addition of a hot, saturated alcoholic solution of picrolonic acid precipitates the histidine practically quantitatively as the histidine picrolonate.

The filtrate from the histidine, containing the arginine silver, is saturated with powdered barium hydroxide, precipitating arginine silver. This precipitate is filtered off, the silver removed with hydrogen sulfide, the excess of barium quantitatively removed with sulfuric acid, and the aqueous solution of arginine concentrated to a small volume and the picrolonate prepared as in the case of histidine.

The solution containing the lysine is freed from silver with hydrogen sulfide, acidified with sulfuric acid, and the lysine precipitated as lysine phosphotungstate. This precipitate is decomposed with baryta, the excess of barium is quantitatively removed with either carbon dioxide or an exact equivalent of sulfuric acid, the solution containing the free lysine is evaporated to a small volume, and the lysine separated as lysine picrate.

This method has been modified in some particulars by later investigators. It is sufficient for our purposes to refer to the paper of Vickery and Leavenworth[39] who have controlled the separation of arginine and histidine silver by hydrogen ion concentration measurements. They point out that histidine silver is completely precipitated at a *p*H of 7.0, whereas arginine silver remains completely in solution. These authors recommend double precipitation, thus insuring the absence of traces of the other amino acids. They likewise recommend the separa-

[39] Vickery, H. B., and Leavenworth, C. S., Modifications of the Method for the Determination of the Basic Amino Acids of Proteins. The Bases of Edestin, *J. Biol. Chem.*, 76 : 707–722 (1928).

tion of arginine as arginine 2.4-dinitro-1-naphthol-7-sulfonate (cf. Pratt).[40]

As already noted, Kossel's method can be made approximately quantitative. Its great *advantage* lies in the fact that by the use of this method the preparation of the basic amino acids can be rather readily accomplished.

4. *The Carbamate Method.*—Buston and Schryver,[41] in 1921, announced a new method for the separation of amino acids from the products of protein hydrolysis. The method depends upon the formation of the carbamino derivatives which we have already discussed. The solution containing the amino acids is treated with an excess of barium hydroxide and saturated with carbon dioxide, forming the barium carbamates. Buston and Schryver observed that the barium salts of the carbamates of the dicarboxylic acids were precipitated by the addition of alcohol. Later Kingston and Schryver[42] studied these reactions and proposed a general scheme for the separation of the hydrolytic products of proteins, based on the carbamate reaction. They find that the dicarboxylic acids can be quantitatively precipitated as the barium carbamates, when three volumes of alcohol are added to the amino-acid solution which has been treated with barium hydroxide and carbon dioxide. When the amino-acid solutions are sufficiently concentrated, practically all of the amino acids can be precipitated in the presence of alcohol, as the barium carbamates, with the exception of proline which remains in the alcoholic mother liquor.

The carbamate method offers particular advantages for the separation of the dicarboxylic acids from the monocarboxylic acids. Very few workers have used the carbamate method. Undoubtedly it deserves more extensive use and investigation.

5. *The Separation of the Basic Amino Acids and the Dicarboxylic Amino Acids by Electrodialysis.*—Foster and Schmidt[43, 44] have successfully utilized the method of electrodialysis to separate the diamino

[40] Pratt, A. E., The Preparation of *d*-Arginine Carbonate, *J. Biol. Chem.*, 67 : 351–356 (1926).

[41] Buston, H. W., and Schryver, S. B., A Method for the Separation of Amino-Acids from the Products of Hydrolysis of Proteins and Other Sources, *Biochem. J.*, 15 : 636–642 (1921).

[42] Kingston, H. L., and Schryver, S. B., Investigations on Gelatin. Pt. III. The Separation of the Products of Hydrolysis of Gelatin by the Carbamate Method, *Biochem. J.*, 18 : 1070-1078 (1924).

[43] Foster, G. L., and Schmidt, C. L. A., The Separation of the Hexone Bases from Certain Protein Hydrolysates by Electrolysis, *J. Biol. Chem.*, 56 : 545–553 (1923).

[44] Foster, G. L., and Schmidt, C. L. A., The Separation of the Dicarboxylic Amino Acids from Certain Protein Hydrolysates by Electrical Transport, *J. Am. Chem. Soc.*, 48 : 1709–1714 (1926).

and the dicarboxylic amino acids from the monoamino monocarboxylic acids. They make use of an electrodialyzing apparatus similar to that shown in Fig. 24. The protein is hydrolyzed with sulfuric acid, the sulfuric acid quantitatively removed with baryta, and the aqueous solution of the amino acids is placed in the center compartment separated from the anode and the cathode chambers by collodion or parchment membranes. Using carbon electrodes and adjusting the acidity of the protein hydrolysate to a *p*H of 5.5, they were able to cause the migration of arginine, histidine, and lysine almost quantitatively to the cathode compartment. At a *p*H of 5.7 only arginine and lysine migrated to the cathode, the histidine remaining in the center compartment. By repeating the electrolysis on the cathode liquor, they were able to separate the basic amino acids almost completely from the other amino acids of the protein hydrolysate. The dicarboxylic acids and proline migrated to the anode, and when the solution in the anode compartment was again electrodialyzed, only small quantities of monoamino monocarboxylic acids were found in the anode compartment. This method of electrolytic migration could probably be used with advantage in a preliminary separation of the diamino acids and the dicarboxylic acids from a protein hydrolysate, as an adjunct to either the Fischer, Dakin, or Kossel method.

6. *The Preparation and Determination of Cystine.*—Cystine can be rather readily prepared from proteins, such as hair or wool, which contain a high percentage of cystine. The method usually employed is that of hydrolyzing the protein with acid, concentrating the mixture of amino acids, and neutralizing the free hydrochloric acid, preferably completing the neutralization with sodium acetate which acts as a buffer, thus avoiding the presence of free alkali which very rapidly decomposes cystine. This method is essentially that proposed by Folin.[45] He recommends that sodium acetate be added to the hydrochloric acid hydrolysate until all of the hydrochloric acid has been replaced with acetic acid. The hydrogen ion concentration at this point is sufficiently reduced so that cystine spontaneously crystallizes, due to its very low solubility. Gortner and Hoffman[46] note that concentrated commercial sodium hydroxide solution can replace to a very large extent the more expensive sodium acetate, providing that the addition of sodium hydroxide is stopped before complete neutralization occurs, the free hydrochloric acid being removed by a final addition of sodium acetate.

Cystine is very readily altered by alkalies, with the exception of

[45] Folin, O., On the Preparation of Cystin, *J. Biol. Chem.*, 8 : 9–10 (1910).

[46] Gortner, R. A., and Hoffman, W. F., *l*-Cystine, Organic Syntheses, Vol. V, pp. 39–41, John Wiley and Sons, Inc., New York (1925).

ammonia. Even washing the hair with a hot 1 per cent solution of sodium carbonate will prevent the isolation of any appreciable amount of cystine from such material. (Cf. Hoffman.[47])

We have already noted that Van Slyke's nitrogen distribution method yields low results for cystine. Okuda[48] proposes the estimation of cystine by oxidation with standard potassium bromate solution. A solution of cystine in 10 per cent hydrochloric acid is treated with 0.1 N potassium bromate in the presence of potassium bromide, the cystine being oxidized to cysteic acid according to the following reactions:

$$KBrO_3 + 5\ KBr + 6\ HCl = 6\ KCl + 3\ H_2O + 3\ Br_2$$

$$\underset{\text{cystine}}{C_6H_{12}O_4N_2S_2} + 10\ Br + 6\ H_2O = 2\ HOOC{-}\underset{\underset{\underset{\underset{\text{cysteic acid}}{}}{}}{NH_2}}{CH}{-}CH_2{-}SO_2{-}OH + 10\ HBr$$

Okuda notes that of all the amino acids precipitated by phosphotungstic acid, histidine is the only one that interferes in the above titration and that the rate of reaction of histidine with bromine is much slower than is the rate of reaction of cystine, so that histidine does not interfere to any great extent.

7. *The Preparation of Tyrosine.*—Due to its relative insolubility in water (1 : 2450 at 17°), tyrosine can be rather readily isolated by direct crystallization. The protein is hydrolyzed with sulfuric acid, the sulfuric acid quantitatively removed with baryta, and the precipitated barium sulfate thoroughly washed with hot water. The filtrates are concentrated to a small volume, and the tyrosine allowed to crystallize. The crude crystals are recrystallized from hot water.

The above methods represent those methods which are usually employed either to isolate individual amino acids or to determine the various groups of amino acids which are present in proteins. In addition there are a number of colorimetric methods which have been proposed and which are rather generally utilized for the determination of individual amino acids. Thus, methods are available for the colorimetric determination of tyrosine, cystine, tryptophane, histidine, and arginine. A description of such methods can be found in most laboratory manuals dealing with physiological chemistry and in particular those dealing with blood analysis. It is felt sufficient to indicate at this point merely the fact that such methods are available.

[47] Hoffman, W. F., Sulfur in Proteins. II. The Effect of Mild Alkaline Hydrolysis upon Hair, *J. Biol. Chem.*, 65 : 251–254 (1925).

[48] Okuda, Y., A Method for the Determination of Cystine, *J. Coll. Agr., Imp. Univ.* Tokyo, 7 : 69–76 (1919).

CHAPTER XIV

PROTEIN STRUCTURE AND THE POSSIBILITIES OF PROTEIN ISOMERISM

We have already noted that the peptide linkage has been definitely proven to be a major linkage in proteins. We have also indicated that polypeptides which have been obtained by the partial hydrolysis of proteins have been artificially synthesized, so that their structure is known, thus indicating that amino acids do occur in the protein molecule bound together in chains by the peptide linkage uniting the amino and carboxyl group.

Within the last few years a number of workers have laid emphasis on the properties of other forms of linkage than that of the long-chain polypeptide type. We cannot discuss any of these viewpoints at length, but it seems worthwhile to indicate the trend of thought in this field.

Vickery and Osborne[1] have presented a critical review of the various hypotheses which have been proposed to account for protein structure. While they are possibly ultra-conservative, nevertheless they have made a real contribution in bringing together a number of divergent views and in comparing them one with another. The author believes that the evidence which has been presented by Abderhalden for the presence of diketopiperazine rings in proteins amounts almost to proof.

1. *Kossel's "Protamine Nucleus" Hypothesis.*—Kossel[2] postulated from his study of the diamino acids that the nucleus of all proteins would be found to be of the "protamine" type, *i.e.*, to be largely composed of either arginine, histidine, or lysine, or combinations of these three amino acids. The protamines, as we shall see later, are probably the simplest proteins and are apparently the characteristic proteins of chromatin. Kossel's hypothesis has been more or less ignored during the more recent investigations. Larmour,[3] however, again calls attention to this

[1] Vickery, H. B., and Osborne, T. B., A Review of Hypotheses of the Structure of Proteins, *Physiol. Rev.*, 8 : 393–446 (1928).

[2] Kossel, A., Ueber die basischen Stoffe des Zellkerns, *Z. physiol. Chem.*, 22 : 176–187 (1896).

[3] Larmour, R. K., Correlations between the Total Nitrogen of the Bases and Arginine Nitrogen and between Total Nitrogen of the Bases and Lysine Nitrogen, of Various Proteins, *Trans. Roy. Soc.*, Canada, Section V, 349–363 (1928).

hypothesis in a statistical study of the relationship which exists between the total basic nitrogen and the arginine nitrogen or the lysine nitrogen of the various proteins, as determined by Van Slyke's method. From a statistical study of 214 analyses of proteins by Van Slyke's method, he finds a coefficient of correlation of $r = +0.794 \pm 0.017$ between the total basic nitrogen and the arginine nitrogen, and a coefficient of correlation between the total basic nitrogen and the deviation of the arginine nitrogen from its most probable value, the mean, of $r = +0.211 \pm 0.044$. The coefficient of correlation between total basic nitrogen and lysine nitrogen for 213 cases was found to be $r = +0.548 \pm 0.032$, and the coefficient of correlation between total basic nitrogen and the deviation of lysine from its most probable value, the mean, was found to be $r = +0.03 \pm 0.046$. Larmour believes that the high correlations in the case of arginine are evidence in support of Kossel's hypothesis that arginine is the nucleus of the protein molecule and states, "It seems highly probable that in all proteins there is a direct and marked relationship between the arginine and the total basic nitrogen."

2. *Abderhalden's Anhydride Theory.*—Abderhalden and his students have during the last few years been emphasizing the probability that proteins are to a very considerable extent built up of cyclic substituted diketopiperazine rings held together by the force of latent valency. The literature which Abderhalden and his students have presented is altogether too voluminous to be cited in detail. Most of the papers will be found in the *Zeitschrift für physiologische Chemie.*

Abderhalden has presented, however, several papers[4-6] in which he sums up certain of the essential theories. From a study of diketopiperazines which have been prepared synthetically, Abderhalden finds that their behavior toward certain chemical reagents is much more nearly like the behavior of proteins toward these reagents than is the behavior of straight-chain polypeptides. Abderhalden has likewise developed certain color reactions, notably that given by picric acid and alkali and that given by meta-dinitro-benzene in alkaline solution, which are characteristic of diketopiperazines, and finds that proteins and peptones of high molecular weight give the characteristic color reaction, whereas polypeptides do not. He has likewise separated from the partial hydrolytic products of proteins a number of diketopiperazines.

[4] Abderhalden, E., Das Eiweiss als eine Zusammenfassung assoziierter, Anhydride enthaltender Elementarkomplexe, *Naturwissenschaften*, 12 : 716–720 (1924).

[5] Abderhalden, E., Über die Struktur der Proteine, *Z. physiol. Chem.*, 128 : 119–128 (1923).

[6] Abderhalden, E., and Schwab, E., Über die Anhydridstruktur des Seidenfibroins, *Z. physiol. Chem.*, 139 : 169–180 (1924).

Brill[7] has investigated the X-ray diffraction pattern of certain proteins, notably silk fibroin, and reaches the conclusion that the basic structure consists of four molecules of glycyl-glycine combined in a ring form, probably as an anhydride. This would tend to support the anhydride ring structure of Abderhalden, and would be in line with the more or less generally accepted view as to the structure of the units making up cellulose fiber.

Abderhalden[8] points out that the presence of an anhydride structure possessing keto $\rightleftharpoons$ enol isomerism may well be responsible for the extreme lability of certain proteins. Abderhalden notes that even in Fischer's octadecapeptide or in his own nonadecapeptide, the polypeptide showed a great stability, was not readily altered or affected by chemical reagents which would not hydrolyze it, and he contrasts this behavior with the behavior of vital proteins. Certain of his comments are so important to the student of vital phenomena that it seems worthwhile to append them in the form of a rather free translation. Thus he states, " Each conception in regard to particular structural relations in proteins, and especially of those which are concerned in living processes, must take into consideration all their reactions, their ready transformation from the natural to the denatured condition, and their greater or less lability. It is certain that proteins in protoplasm have properties of which we are at present entirely unaware. We study proteins almost invariably in a greater or a less changed condition. On the one hand, we have proteins which in a certain sense are denatured in the organism, and outside of the organism undergo further changes. Thus, for example, we see the silk threads, the web of the spider, the byssus of certain mussels, etc., which are extruded as a liquid, changing to an inert solid. From a very labile form they are transformed into a very stable structure. In living processes we deal with the very reactive cell proteins which regulate in a fine degree the reactions of the cell, but when death ensues, the cell proteins coagulate and lose in a large measure their characteristic physical behavior."

These comments of Abderhalden cannot be too strongly emphasized to those working in the field of the proteins, and in order to account for the reactivity of cell proteins we must postulate linkages which are more labile than are the linkages represented by the peptide structure alone. Incidentally it should be noted again that Fischer[9] recognized the pos-

[7] Brill, R., Über Seidenfibroin, I., *Ann.*, 434 : 204–217 (1923).

[8] Abderhalden, E., and Komm, E., Über die Anhydridstruktur der Proteine, *Z. physiol. Chem.*, 139 : 181–204 (1924).

[9] Fischer, E., Untersuchungen über Aminosäuren, Polypeptide und Proteine, *Ber.*, 39 : 530–610 (1906).

sibility that diketopiperazine rings, such as Abderhalden has been emphasizing, might well be present in the protein molecule.

3. *Bergmann's Hypothesis.*—Bergmann has formulated a hypothesis in which he postulates protein structure as being composed of piperazine derivatives held together by forces of latent valency. His theory is very similar to that of Abderhalden, with the exception that Bergmann[10, 11] has been particularly interested in the reactions of hydroxy amino acids and their effect upon the ring structure. The series of compounds on the following page illustrates Bergmann's viewpoint.

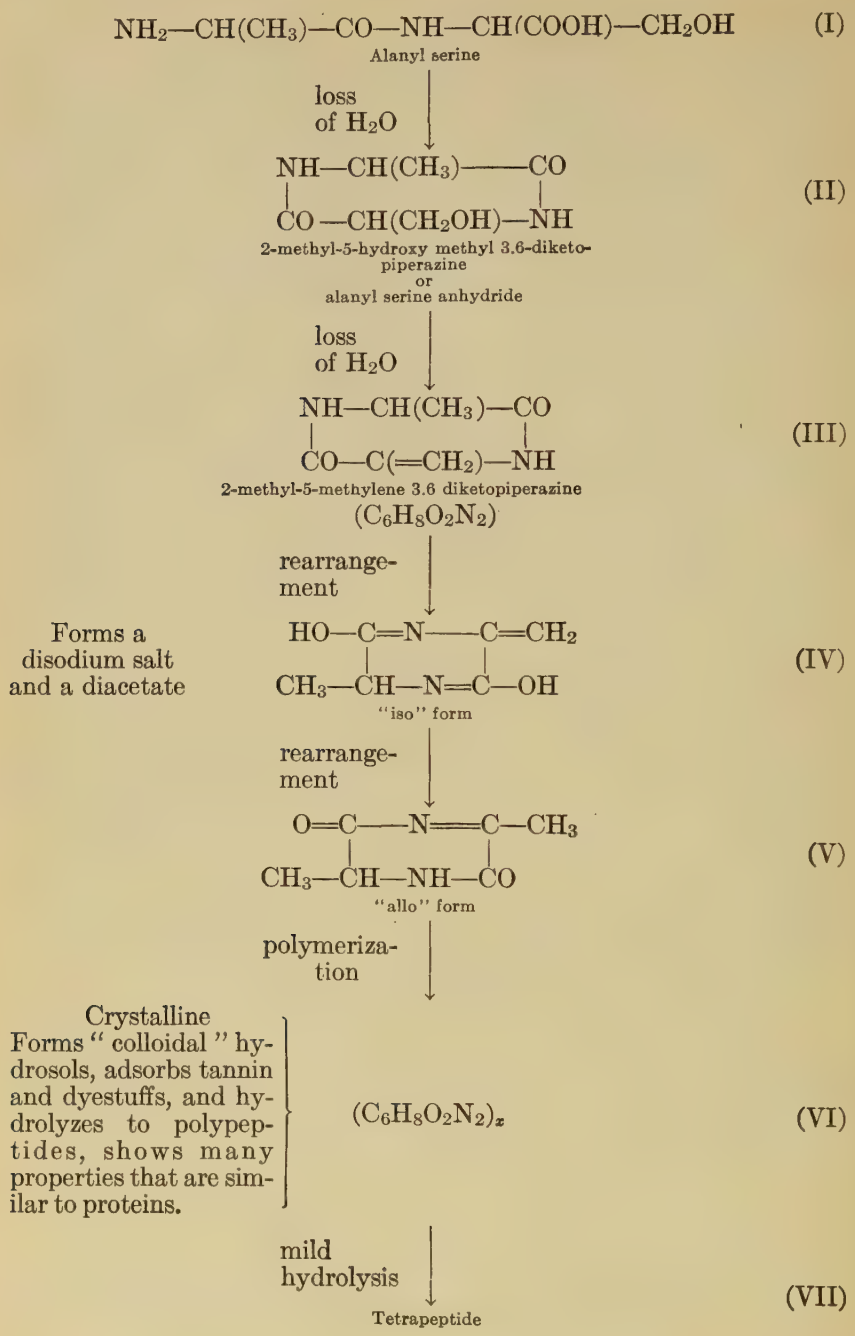
It will be noted that compound (II) is the ordinary dipeptide anhydride or diketopiperazine. Bergmann finds that such compounds formed from hydroxy acids lose a further molecule of water, yielding methylene derivatives similar to (III), and that these undergo rearrangement with various reagents to form compounds, such as (IV) and (V) which he characterizes as the " iso " and " allo " forms, respectively, the " allo " form apparently polymerizing so that in the presence of water it yields a colloidal hydrosol which adsorbs tannin and a number of dyestuffs, and the polymerized product in many of its reactions resembles the reactions of protein.

Bergmann points out that none of the simple polypeptides or the simple diketopiperazines adsorb tannin in appreciable amount, whereas his polymerized " allo " form is strikingly like the proteins in that regard. Furthermore, when the " allo " form was subjected to mild hydrolysis, he isolated not the original dipeptide but rather a tetrapeptide which led him to suggest that the isolation of tetrapeptides from proteins is not necessarily proof that the tetrapeptide existed preformed in the protein molecule but may have been derived from some cyclic structure analogous to his polymerized " allo " form. He suggests that the " allo " form polymerizes through secondary valence forces which are resistant toward water or ordinary aqueous solutions.

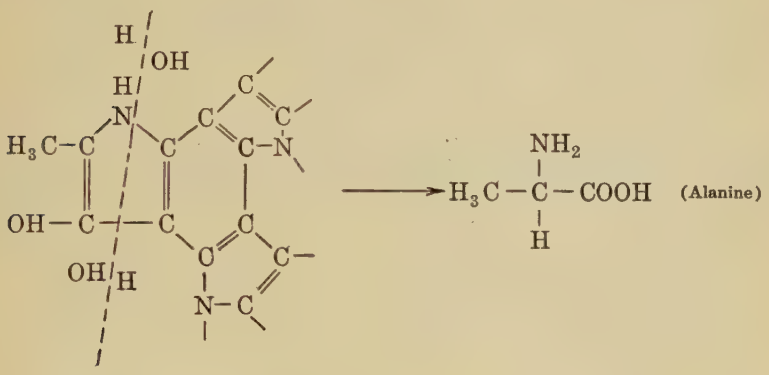
Bergmann admits that no definite proof exists for the presence of such compounds in the protein molecule but believes that the hydroxy amino acids would undergo similar reactions and that it is by studying such a series of synthetic compounds as he has prepared that we can advance the theories of protein structure.

[10] Bergmann, M., Über neuere Proteinchemie, *Naturwissenschaften,* 12 : 1155–1161 (1924).

[11] Bergmann, M., Über den hochmolekularen Zustand der Proteine und die Synthese proteinähnlicher Piperazin-Abkömmlinge, *Naturwissenschaften,* 13 : 1045–1050 (1925). *Cf.* also numerous papers in the *Annalen* and in *Zeitschrift für physiologische Chemie.*


NH2—CH(CH3)—CO—NH—CH(COOH)—CH2OH
(I)
Alanyl serine
loss of H2O
NH—CH(CH3)——CO
CO—CH(CH2OH)—NH
(II)
2-methyl-5-hydroxy methyl 3.6-diketo-piperazine
or
alanyl serine anhydride
loss of H2O
NH—CH(CH3)—CO
CO—C(=CH2)—NH
(III)
2-methyl-5-methylene 3.6 diketopiperazine
(C6H8O2N2)
rearrange-ment
Forms a disodium salt and a diacetate
HO—C=N——C=CH2
CH3—CH—N=C—OH
(IV)
"iso" form
rearrange-ment
O=C——N===C—CH3
CH3—CH—NH—CO
(V)
"allo" form
polymeriza-tion
Crystalline
Forms " colloidal " hydrosols, adsorbs tannin and dyestuffs, and hydrolyzes to polypeptides, shows many properties that are similar to proteins.
(C6H8O2N2)x
(VI)
mild hydrolysis
Tetrapeptide
(VII)

4. *Troensegaard's Pyrrole Hypothesis.*—Troensegaard[12] has developed a theory of protein structure based largely upon a study of completely acetylated proteins and the hydrolytic products of such proteins. He postulates the presence of heterocyclic rings, largely of the pyrrole type, as indicated in the following diagram:



In this diagram Troensegaard postulates hydrolysis as occurring along the dotted line, liberating alanine as one of the hydrolytic products. As Vickery and Osborne point out, such a structure as Troensegaard postulates would not appear to be the normal structure characteristic of proteins for two reasons, (1) the double bond in the condensed alanine structure should, when hydrolyzed according to Troensegaard's scheme, yield a racemic mixture of amino acids rather than a single optically active acid, and (2) the proposed ring system contains an arrangement of six carbons identical with the arrangement of carbons in the benzene ring. Accordingly such a structure should be very stable when submitted to acid hydrolysis.

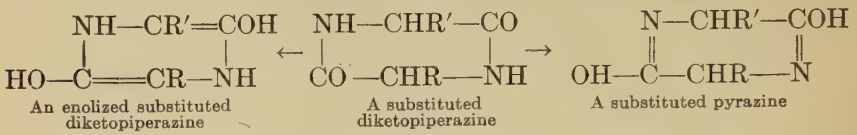
In spite of the above criticisms, Troensegaard has introduced new technic into protein research and has presented sufficient evidence so that future investigators must take into consideration the possibility that pyrrole ring structures may well occur in the protein molecule.

5. *Karrer's Hypothesis.*—Karrer[13-15] has called attention to the

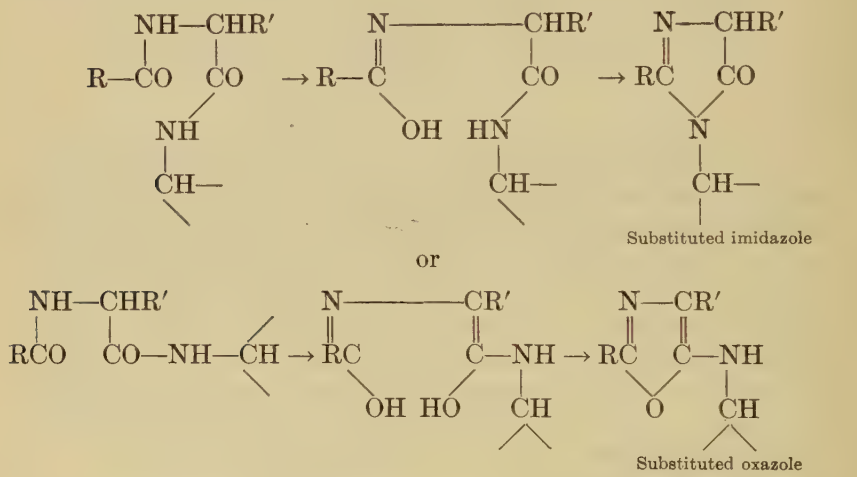
[12] Troensegaard, N., Über die Konstitution der Eiweissverbindungen, *Z. angew. Chem.*, 38 : 623–626 (1925); cf. also *Z. physiol. Chem.*, 112 : 86–103 (1920); 127 : 137–185 (1923); 130 : 84–86 (1923); 133 : 116–125 (1924); 134 : 100–112 (1924); 142 : 35–70 (1925); 153 : 93–110 (1926).

[13] Karrer, P., and Gränacher, C., Über Anhydride von Aminosäuren und Aminosäurederivaten, *Helv. Chim. Acta*, 7 : 763–780 (1924).

possibilities of the enolization of diketopiperazines and of structures which may be formed by the enolization of polypeptides. Thus, diketopiperazine may enolize in two ways, the hydrogen being withdrawn from the imino groups or from attachment to the adjacent carbon.



In either case the ring structures which result should have reactions different from those of the diketopiperazine or of normal polypeptides. Similarly, polypeptides may be considered to enolize according to the following scheme:



yielding substituted imidazoles or substituted oxazoles. Aside from the occurrence of the imidazole ring in histidine, the presence of imidazoles or oxazoles in the normal structure of proteins still remains to be proven.

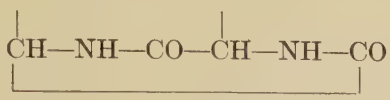
6. *The Peptine Ring Hypothesis.*—Ssadikow and Zelinsky[16] have presented a further hypothesis which they characterize as a peptine ring.

[14] Karrer, P., and Widmer, R., Zur Kenntnis der Aminosäuren, *Helv. Chim. Acta*, 8 : 203–205 (1925). (Cf. also 8 : 205–211 and 211–217.)

[15] Karrer, P., Gränacher, C., and Schlosser, A., Zur Kenntnis der Diketopiperazinderivate, *Helv. Chim. Acta*, 6 : 1108–1112 (1923).

[16] Ssadikow, W. S., and Zelinsky, N. D., Über Produkte der katalytischen Spaltung von Eiweissstoffen, *Biochem. Z.*, 136 : 241–249 (1923).

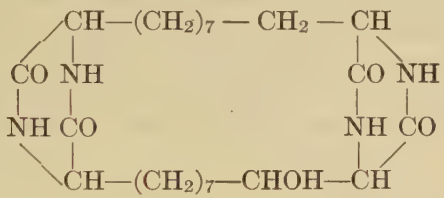
They are in general agreement with the other workers who insist that the constitution of the protein molecule is not that of a long polypeptide chain but rather is that of a system of cyclic structures. They propose a " polypeptine " unit with its basis as the peptine ring, *i.e.*, a substituted piperazine,



The " peptine ring "

the only difference from the diketopiperazine theory being that they suggest that these rings are held together not by secondary valences but by long hydrocarbon chains and that cyclic or heterocyclic rings may be attached to the side chains.

The essence of their theory is that the linkage between the carboxyl and the amino groups forms cyclic compounds rather than straight chain compounds and that the ring structures which are formed, in general may contain more than one diketopiperazine ring, as is indicated in the following structure which they believe represents a compound that they isolated by the partial hydrolysis of proteins.

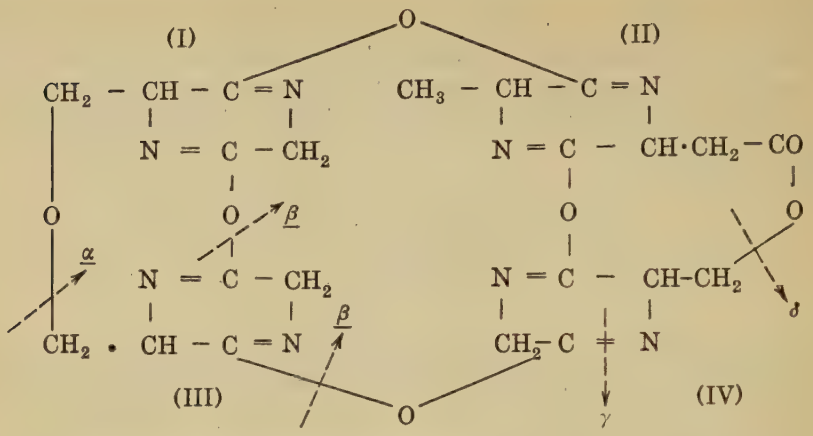


Their proof that this is the structure is not conclusive.

In a later paper, Ssadikow[17] suggests different types of ring structures as characterizing the behavior of different proteins toward enzymes. Here again, the paper must be regarded largely as theoretical. On the the other hand, theory may well precede demonstration, providing that it is recognized as theory and used as a hypothesis which may interpret experimental data. He divides the proteins into four classes.

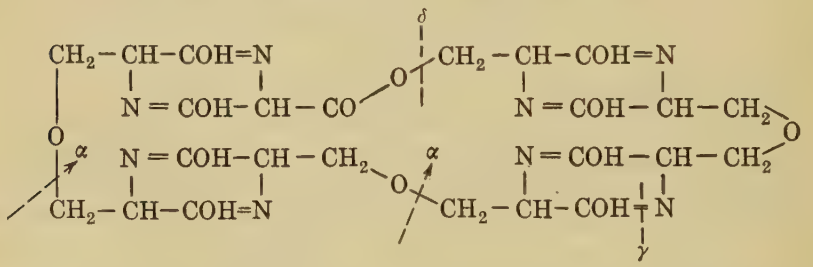
The *collagen type* is characterized as being readily digested by pepsin but resistant to the action of trypsin, and he suggests that this protein may be built up of four peptine rings, as shown in the following diagram:

[17] Ssadikow, W. S., Über einige Bindungsarten im Eiweissmolekül, *Biochem. Z.*, 179 : 326–331 (1926).



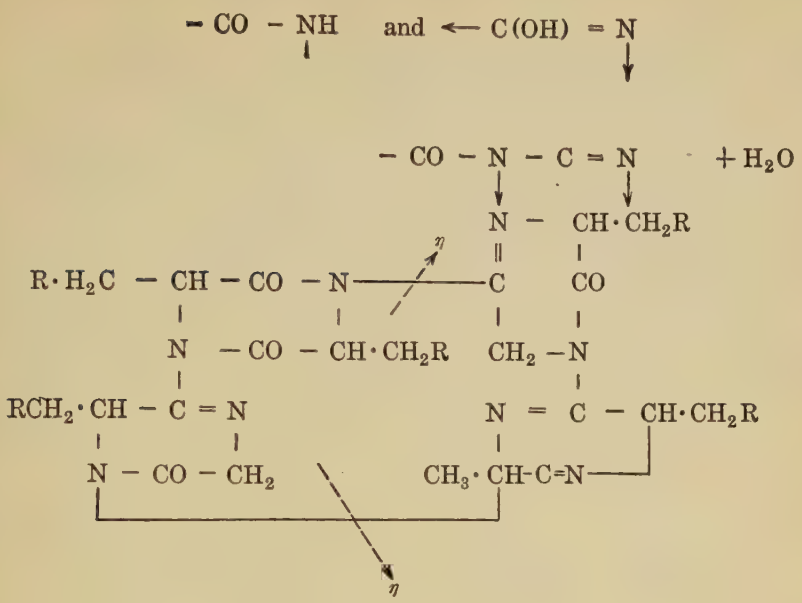
The union at α is formed between two —CH_2OH groups with the elimination of water, the union at β between two —C(OH) groups with the elimination of water, the union at δ between a —CH_2OH group and a —COOH group with the elimination of water, and the union at γ as representing an enolized peptide linkage. It is pointed out that the α, β, and δ unions may be considered as being hydrolyzed by pepsin and not hydrolyzed by trypsin, and while normally the peptide linkage at γ would be hydrolyzed by trypsin, the fact that it is enolized and the enolic group united through a β linkage makes it trypsin stable.

The *gelatin type* of protein is hydrolyzed by both trypsin and pepsin. He represents these linkages diagrammatically as follows:

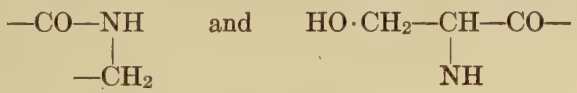


pepsin hydrolizing the α and δ linkages, and trypsin hydrolyzing the γ linkage which is not blocked in this case, no β linkages being present.

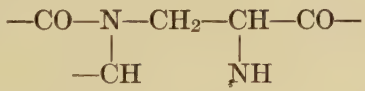
The *keratin type* of protein is stable to both pepsin and trypsin. He interprets this to mean that there are no α, β, or δ linkages and that the γ linkage is blocked, but not through a β union. He accordingly postulates a η linkage through a nitrogen atom, all three valences of which are attached to carbon.



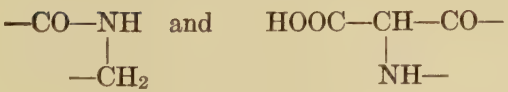
Ssadikow points out that this union may be accomplished in two ways, both involving the loss of the elements of water. The ν and μ linkages are shown as follows:



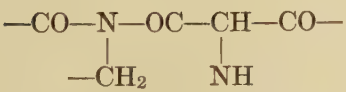
give



(ν linkage)

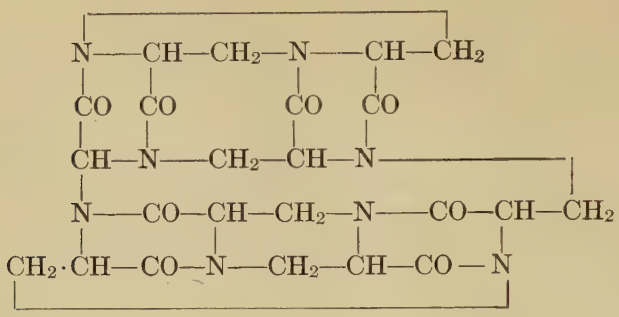


give



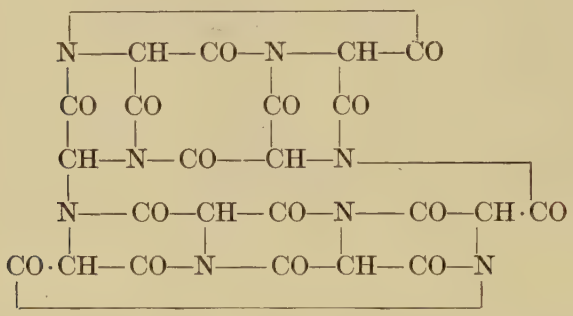
(μ linkage)

Four such peptine rings could be considered as uniting through these linkages to form structures of the following type:



(ν linkage)

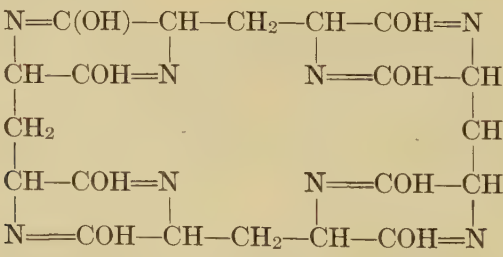
or



(μ linkage)

The ν- and μ-linkages he regards as resistant to both pepsin and trypsin.

The *peptone type*, pepsin-resistant but easily hydrolyzable by trypsin, he regards as containing no α, β, and δ linkages, and the γ linkages as not being blocked. One would thus obtain a structure possibly of the following type:

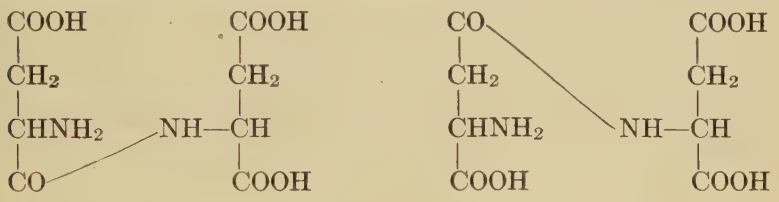


(ψ-linkage)

It should be noted that in the above structural diagrams, Ssadikow has made use of the simple diketopiperazine ring rather than the substituted diketopiperazine rings, so that if one were to extend his argument into the field of protein structure, most of the hydrogen atoms in the structural formulae would have to be replaced by aryl or alkyl radicals. While admittedly the entire argument presents a hypothesis,

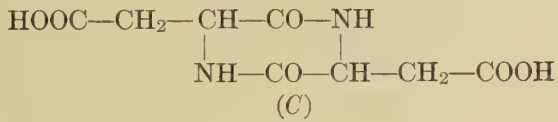
nevertheless insofar as the author is aware, this is the first attempt to explain the specific reactions of proteins toward trypsin and pepsin in terms of structural organic chemistry, and it is for that reason that the hypothesis is reproduced.

In addition to the above theories, it seems wise to point out here one other transformation involving ring structures, which appears to be characteristic of the dipeptides of aspartic and glutamic acids. Blanchetière,[18] in studying the behavior of dipeptides of aspartic and glutamic acids, isolated two products, one of which appears to be the normal dipeptide in which the peptide linkage is through the carboxyl and amino groups attached to the α carbon atoms, as shown in (*A*). The other or ω peptide is characterized by the peptide linkage being formed by the union of the α-amino with a β-carboxyl group, as shown in (*B*).

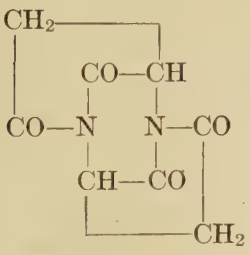


(*A*) (*B*)

Both these types of dipeptides were synthesized. When these two forms were converted into anhydrides, one formed a normal substituted diketopiperazine, as shown in (*C*), the other a system of three condensed rings, as shown in (*D*).



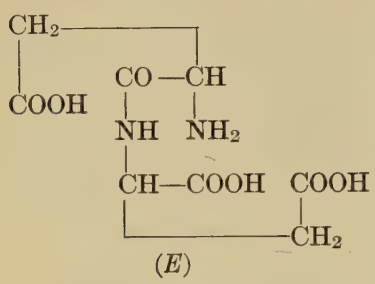
(*C*)



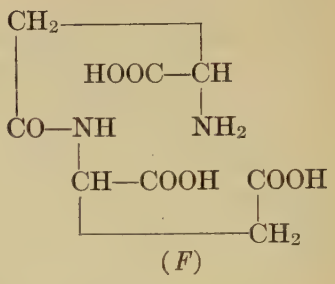
(*D*)

[18] Blanchetière, M. A., Constitution des anhydrides des acides aspartique et glutamique. Son importance biologique, *Bull. soc. chim. biol.*, 6 : 854–860 (1924).

Compound (*D*) undergoes hydrolysis, yielding two types of dipeptides (*E*) and (*F*).

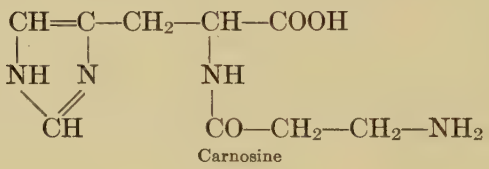


(*E*)



(*F*)

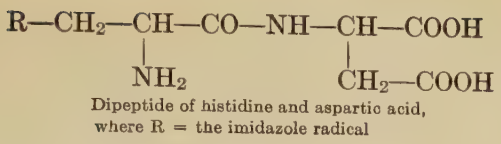
We have already noted that Engeland isolated, in 1908, a β-amino acid, β-alanine. This was found in the liquid expressed from muscular tissue. Blanchetière points out that while β-amino acids have not been found to occur in proteins, they do occur in other compounds characteristic of living material, such as carnosine (β-alanyl histidine).



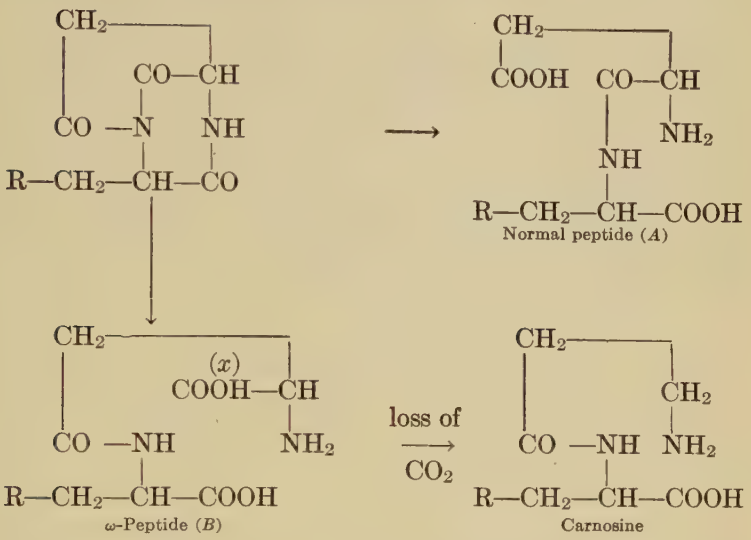
Carnosine

He accordingly notes that carnosine in muscle cannot originate from ordinary alanine (α-amino propionic acid) and histidine, but that it must originate in some other way. The mechanism which he proposes is an initial dipeptide between histidine and aspartic acid, which in turn condenses to an internal anhydride capable of hydrolyzing to yield two different compounds, peptide (*A*) and peptide (*B*). Peptide (*A*) is the normal peptide which one would expect between histidine and aspartic acid. Peptide (*B*) is abnormal. He notes that peptide (*B*) is very sensitive to reagents and readily undergoes decarboxylation, losing carbon dioxide at (*x*), to yield carnosine.

Blanchetière points out that the ω-peptides are apparently all characterized by the great ease with which they lose carbon dioxide. The fact that Blanchetière has prepared the double anhydrides containing three condensed rings is at least suggestive that such compounds may occur more generally in nature than we have believed.



Dipeptide of histidine and aspartic acid, where R = the imidazole radical



Normal peptide (*A*)

ω-Peptide (*B*)

Carnosine

The Possibilities of Protein Isomerism.—We have already noted in a consideration of the polypeptides that there are six possible isomers of the tripeptide containing tyrosine, leucine, and alanine, and that in order to identify a naturally-occurring tripeptide containing these amino acids, it would be necessary to synthesize the various isomers to determine the space configuration of the polypeptide which had been isolated.

The number of possible isomers of a tripeptide is accordingly $\underline{|3}$, which is equal to $3 \times 2 \times 1$. For a tetrapeptide we would have $\underline{|4}$, or $4 \times 3 \times 2 \times 1$, or 24 possible isomers.

Assuming a protein to be made up of 20 amino acids, and *assuming that each amino acid occurred only once in the protein chain*, there would still remain the possibility of having $\underline{|20}$ or approximately 23×10^{17} different compounds, each containing the same amino acids in identical proportions and differing only in space relationships.

According to modern viewpoint, proteins have a very much greater molecular weight than would be found for such a " polypeptide." Egg albumin, according to the best evidence, appears to have a molecular weight of about 34,000, so that presumably an egg albumin molecule is

made up of several hundred molecules of individual amino acids. Regardless of how these are linked together, it must be obvious from the above that there are limitless theoretical possibilities to the number of compounds which could be synthesized from the known list of amino acids. The author has been emphasizing these possibilities for a number of years.

Leathes[19] recently has called attention to these possibilities in a very striking way. He states, "Supposing it were a chain of only 50 links, a very simple case; if all the links were different the number of possible permutations is denoted by the innocent-looking symbol $\lfloor\underline{50}$. If, instead of all being different, one kind of link recurred ten times, the number would be reduced to $\lfloor\underline{50} / \lfloor\underline{10}$. If, in addition, there were 4 that recurred four times and 10 that recurred twice, it would be further reduced to

$$\lfloor\underline{50} / \lfloor\underline{10} \times (\lfloor\underline{4})^4 \times (\lfloor\underline{2})^{10}.$$

It would now consist of a chain of only 50 links, of which there were only 19 different kinds, and the number of different arrangements of its parts would be about 10^{48}. Astronomy deals with big figures. Light, it is said, takes 300,000 years to travel from one end of the Milky Way to the other; this distance expressed in Ångström units, 10,000,000 of which go to a millimeter, would be less than 10.[32] So far are we from knowing the structure of protein molecules. So far are we from knowing what variations in disposition of the parts in such a molecule may not occur without our being within a measurable distance of detecting them. For if the number of possible varieties of a protein whose molecular weight is known, and known to be exceptionally small, and which contains the several amino acids in a known proportion, is as great as this, the number that is possible when that proportion may be changed is practically incalculable, each change in proportion being capable of a number of new arrangements that could be calculated, as was done for our hypothetical case. . . . . The peculiar thing about the chemistry of living matter is not that the reactions that are characteristic in it are novel, but that in the rough and tumble of ordinary liquid systems their occurrence is almost infinitely improbable. Where there is life circumstances exist which make them the rule. . . . . Forces which determine the relative positions of adjacent foreign molecules and so affect their behavior are something to which there is no analogy in the growth of crystals in a saturated solution."

Leathes' calculations do not take into consideration the possibility

[19] Leathes, J. B., Function and Design, *Science*, 64 : 387–394 (1926). (Every biochemist or biologist interested in vital phenomena should read this paper.)

of various linkages between the various amino acids, keto $\leftrightharpoons$ enol isomerism, cyclic structures, such as have been discussed in the preceding part of this chapter, etc., all of which would introduce further possibilities in calculating the number of possible isomers which could be formed from a given number of amino acids.

As we shall see later, the biological reactions of the proteins enable us to differentiate rather sharply, in most instances, between the proteins which are characteristic of one species and those which are foreign to that species, and it is upon these observations that the entire modern structure of immunology has been built. Some workers point out that the species specificity which is demanded by the modern theories of immunology and which is shown in immunological reactions, is almost infinite and therefore surprising. The author believes with Leathes that, considering the infinite possibilities of the linkages of amino acids in proteins, it is much more surprising that nature should ever synthesize twice in succession proteins which are even remotely alike.

Perhaps no more striking illustration of the exactness with which vital reactions are regulated in the living protoplasm can be given than the fact that, through untold generations, a given organism reproduces the same structural configurations in the proteins which characterize that organism. The fact that each species has a different set of proteins is not the surprising thing. The surprising thing is that nature is able to control the synthesis of proteins within a single species, so that the same protein is synthesized by all members of the species.

CHAPTER XV

PROTEIN CLASSIFICATION

Inasmuch as the exact chemical composition of nearly all of the proteins is as yet unknown, it has been found convenient to divide them into classes, the basis of classification being chemical in so far as is possible, and where a chemical classification is not possible, solubility (or probably better, peptization, *vide infra*) has been made the basis. Two general classifications have been proposed, one by a joint committee of the American Physiological Society and the American Society of Biological Chemists, the other by the English Biochemical Society. These are referred to as the American classification and the English classification.

The American Classification.—In 1908, the committees appointed by the American Physiological Society and the American Society of Biological Chemists recommended the classification[1] which is essentially reproduced below. In a few instances it has seemed wise to slightly change the wording of their definitions or to include additional comments.

I. The Simple Proteins.—These are the naturally-occurring proteins which on being treated with enzymes or acids are hydrolyzed only into α-amino acids or their derivatives. They differ from the conjugated proteins in that the latter are hydrolyzed not only into amino acids but also yield other non-protein substances. Within the group of the simple proteins a number of subdivisions may be recognized, largely on the basis of solubility or other properties.

A. The Albumins.—The albumins are soluble in water and in dilute salt solutions and are coagulable by heat. Typical examples are ovalbumin from egg white, lactalbumin from milk, and vegetable albumins such as the leucosin from wheat. (As a matter of fact, some of the albumins which have been thoroughly studied, notably egg albumin, contain carbohydrate residues and should accordingly be classified strictly with the conjugated proteins under the sub-class of "glycoproteins." Both the American and English classifications, however,

[1] Joint Recommendations of the Physiological and Biochemical Committees on Protein Nomenclature, *J. Biol. Chem.*, 4 : xlviii-li (1908).

list them as the first sub-class of the simple proteins. This fact is an illustration of the difficulties of exact protein classification.)

B. The Globulins.—The globulins are simple proteins, insoluble in pure water, but soluble in dilute neutral solutions of the salts of strong bases and acids. Typical examples which could be cited are the ovoglobulin of egg yolk, the myosin of muscle, and edestin from hempseed. Typical globulins have been isolated from a great many vegetable seeds.

C. The Glutelins.—These are simple proteins, insoluble in all neutral solvents, but readily soluble in *very* dilute acids and alkalies. Examples are the glutenin from wheat, oryzein of rice, etc.

D. The Prolamines or Alcohol-Soluble Proteins.—This class is defined as being insoluble in water, absolute alcohol, or other neutral solvents, but soluble in relatively strong alcohol (70–80 per cent). Typical examples are zein from corn, gliadin from wheat, hordein from barley, etc.; with a single exception (the alcohol-soluble protein from milk, isolated by Osborne) the prolamines are confined to the seeds of the cereal grains.

E. The Albuminoids.—These are defined as simple proteins which possess essentially the same chemical composition as other proteins, but are characterized by a great insolubility in all neutral solvents. They are, in general, insoluble in dilute acid, alkali, water, or salt solutions. This group is in reality a heterogeneous group, containing various proteins of widely different physical properties. Apparently all the proteins which do not fit definitely into some other class are grouped with the albuminoids. Examples are the keratin from horn, hide, hoof, hair, and feathers of animals, elastin from ligaments, collagen from tendons, and gelatin from hide, hoofs, bones, etc. Gelatin, while classed as an albuminoid, does not agree with the definition noted above. It is more properly a protein derived from collagen, which fact probably accounts for its inclusion in the group.

F. The Histones.—Histones are soluble in water, insoluble in very dilute ammonia, not coagulable by heat, easily soluble in very dilute acids, and on acid hydrolysis yield a large number of amino acids, among which the basic amino acids predominate. As a general rule, histones form precipitates with solutions of other proteins. Histones may be regarded as basic proteins, their basicity being intermediate between the protamines and the more common proteins. Typical examples are the globin from hemoglobin and the histones from birds' corpuscles and from the thymus gland.

G. The Protamines.—These are the simplest natural proteins. They are highly basic, soluble in water, soluble in ammonia, form true salts

with mineral acids, many of the salts being crystalline. They are not coagulable by heat, and yield on hydrolysis relatively few amino acids with the diamino acids greatly predominating. They have the property of precipitating other proteins from aqueous solution. They are typically found in the ripe sperm cells and have been largely isolated from fish sperm. Examples are salmin, containing 88 per cent of arginine, from salmon sperm, sturin from sturgeon sperm, etc.

II. Conjugated Proteins.—These are compounds of simple proteins with some other non-protein group, the union with the non-protein molecule being otherwise than as a salt.

A. Nucleoproteins.—These are compounds of one or more protein molecules with nucleic acid. They are the proteins of the cell nuclei and apparently make up in a large measure the substance of the chromatin. Examples are nuclein and nucleohistone from tissues rich in cells, such as glandular tissues, yeast, etc.

B. Glycoproteins or Glucoproteins.—These are proteins in which the additional group is a carbohydrate radical other than in the form of that contained in nucleic acid. The mucus-yielding proteins of tissues are particularly rich in glycoproteins. They possibly serve as a cementing substance for holding together the fibers in tendons and ligaments. The mucin which is secreted by the snail or by the salivary glands, the protein making up the great majority of the structure of the jelly fish, and the proteins forming the jelly surrounding fish eggs and amphibian eggs are typical examples of these proteins.

C. Phosphoproteins.—The additional group in these proteins is not known, but it contains phosphoric acid in some other form than as nucleic acid or as a phospholipide. Typical examples are casein from milk, and vitellin from egg yolk.

D. Chromoproteins.—This group is referred to in the original classification as the *hemoglobins*. It should, however, be extended to include other proteins than the hemoglobins. The conjugated group is colored and may be hematin as in hemoglobin, cyanin as in hemocyanin, or a group of unknown nature, such as occurs in the colored proteins of certain seaweeds where the proteins have been named phycoerythrin and phycocyan. Chromoproteins likewise occur in certain pigmented animal fibers, such as black wool, hair, etc. The colored group is apparently melanin.

E. Lecithoproteins.—These are the proteins of the cytoplasm and of the cell membrane area. The conjugated group is lecithin or a phospholipide. Lung tissue is rich in such proteins.

F. Lipoproteins.—This group was added by A. P. Mathews, the conjugated group being one of the higher fatty acids. Such compounds

are so easily prepared artificially that their natural occurrence is deemed probable.

III. Derived Proteins.—This group is an artificial one but includes the various decomposition products of the naturally-occurring proteins which have been produced by the action of reagents or enzymes or physical agents, such as heat, hydrogen ion concentration, etc. It also includes the artificially synthesized compounds. It is divided into various groups according to solubility, and to a lesser extent according to the degree of complexity.

A. Primary Protein Derivatives.—

1. *Coagulated Proteins.*—These are insoluble protein products produced from natural proteins by the action of heat, alcohol, or by some similar method.

2. *Proteans.*—The initial product of the action of very dilute acids or, in some instances, water or enzymes, upon certain globulins. This form of derived protein is particularly characteristic of the globulins and differs physically from the globulins by a loss of solubility in dilute salt solutions. Examples are edestan from edestin, myosan from myosin, etc.

3. *Metaproteins.*—These are produced by the further action of acids and alkalies upon proteins. They are, as a rule, characterized by being soluble in very weak acids or alkalies but by being insoluble in neutral solutions. Examples are acid metaprotein (acid albuminate), alkali metaprotein (alkali albuminate), etc.

B. Secondary Protein Derivatives.—

1. *Proteoses.*—The partial hydrolytic decomposition products of proteins. These are soluble in water, non-coagulable by heat, and *precipitated* by saturating their solutions with ammonium sulfate.

2. *Peptones.*—The partial hydrolytic decomposition products of proteins. They are soluble in water, non-coagulable by heat and *not precipitated* by saturating the solutions with ammonium sulfate.

3. *Peptides.*—These are definitely characterized compounds of two or more amino acids, the carboxyl group of one being united with the amino group of the other, with the elimination of a molecule of water. They are not heat-coagulable; they may or may not give the biuret reaction; they may be either of natural origin or synthetic.

4. *Diketopiperazines.*—These are cyclic anhydrides of two amino acids. They may be regarded as the anhydride of a dipeptide.

The English Classification.—The English classification is as follows:

I. Simple Proteins
   A. Protamines
   B. Histones
   C. Globulins
   D. Albumins
   E. Glutelins
   F. Gliadins (prolamins) (Soluble in 80 per cent alcohol; insoluble in water.)
   G. Scleroproteins (forming the skeletal structure of animals)
   H. Phosphoproteins

II. Conjugated Proteins
   A. Chromoproteins
   B. Nucleoproteins
   C. Glucoproteins

III. Hydrolyzed Proteins
   A. Metaproteins
   B. Albuminoses or proteoses
   C. Peptones
   D. Polypeptides

The definitions adopted for these groups are essentially those already given in the American classification. It will be noticed that the English classification places the phosphoproteins among the simple proteins.

A casual glance at the above systems of classification indicates definitely that the major distinction lies in physical properties. *Solubility* and *precipitability* have been emphasized at a number of points. Since the above classifications were adopted a great deal of attention has been given to the physico-chemical properties of protein systems. No one appears to have definitely challenged the generally accepted systems of protein classification prior to the recent paper of Gortner, Hoffman, and Sinclair, although many workers have pointed out the fact that changes in solubility could be brought about by various reagents.

Hoffman and Gortner[2] in an attempt to isolate a quantity of the various proteins which had been reported to occur in wheat flour, observed that extracting the flour with 5 per cent potassium sulfate solution and with 10 per cent sodium chloride solution did not yield similar fractions, but that instead, the amount and character of the protein material dissolved by these two reagents were markedly different. These differ-

[2] Hoffman, W. F., and Gortner, R. A., The Preparation and Analysis of the Various Proteins of Wheat Flour with Special Reference to the Globulin, Albumin, and Proteose Fractions, *Cereal Chem.*, 4 : 221–229 (1927).

ences were especially noticeable in the globulin fraction. According to the definition of globulin, the two solutions should have yielded identical fractions.

Accordingly, Gortner, Hoffman, and Sinclair[3] definitely raised the question which is ignored in the definition of globulins, *i.e.*, " simple proteins, heat-coagulable, insoluble in water, but soluble in dilute solutions of the salts of strong acids and bases," and they ask the question, " *What salts, and what concentrations?*" They therefore undertook an extensive study of this question, utilizing wheat flour as the biological material and studying the behavior of 22 different salts, most of them in three or more concentrations.

In their study they used 12 different wheat flours, practically all of which were extracted with the various concentrations of the various salt solutions. Table XLVIII shows the average percentages of protein extracted from this series of wheat flours by the various concentrations of the different salt solutions. The data in Table XLVIII is shown graphically in Fig. 103.

At the same time that these workers were studying the percentage of protein extracted by the salt solution, they likewise studied the hydrogen ion concentration of the various extracts. For our present purpose, we need only to be concerned with the values which were obtained for the 1.0 N solutions of the potassium halides. These solutions were adjusted so as to have an identical hydrogen ion concentration. We have here a lyotropic series of KF < KCl < KBr < KI, with extreme ranges in the percentage of total protein extracted of from 13.07 per cent to 63.89 per cent. *In this instance, at least, the degree of peptization can be due only to a specific influence of the anions which are present in equivalent concentrations.*

It seemed pertinent therefore, in view of the data shown in Table XLVIII and Fig. 103, to definitely raise the question, " What is a globulin?"

The authors point out that in the light of these results, it should be obvious that a definition which is based upon solubility, *i.e.*, peptization, in a dilute salt solution is so ambiguous as to be absolutely meaningless. They further point out that all of the various protein fractions which have been considered to be present in wheat flour and which are regarded as fixed entities can be isolated in a constant proportion, providing that a prescribed routine procedure for protein isolation is followed. If, however, that prescribed routine procedure is altered some-

[3] Gortner, R. A., Hoffman, W. F., and Sinclair, W. B., Physico-Chemical Studies on Proteins. III. Proteins and the Lyotropic Series, Colloid Symposium Monograph, Vol. V, pp. 179–198 (1928); cf. also *Koll. Z.*, 44 : 97-108 (1928).

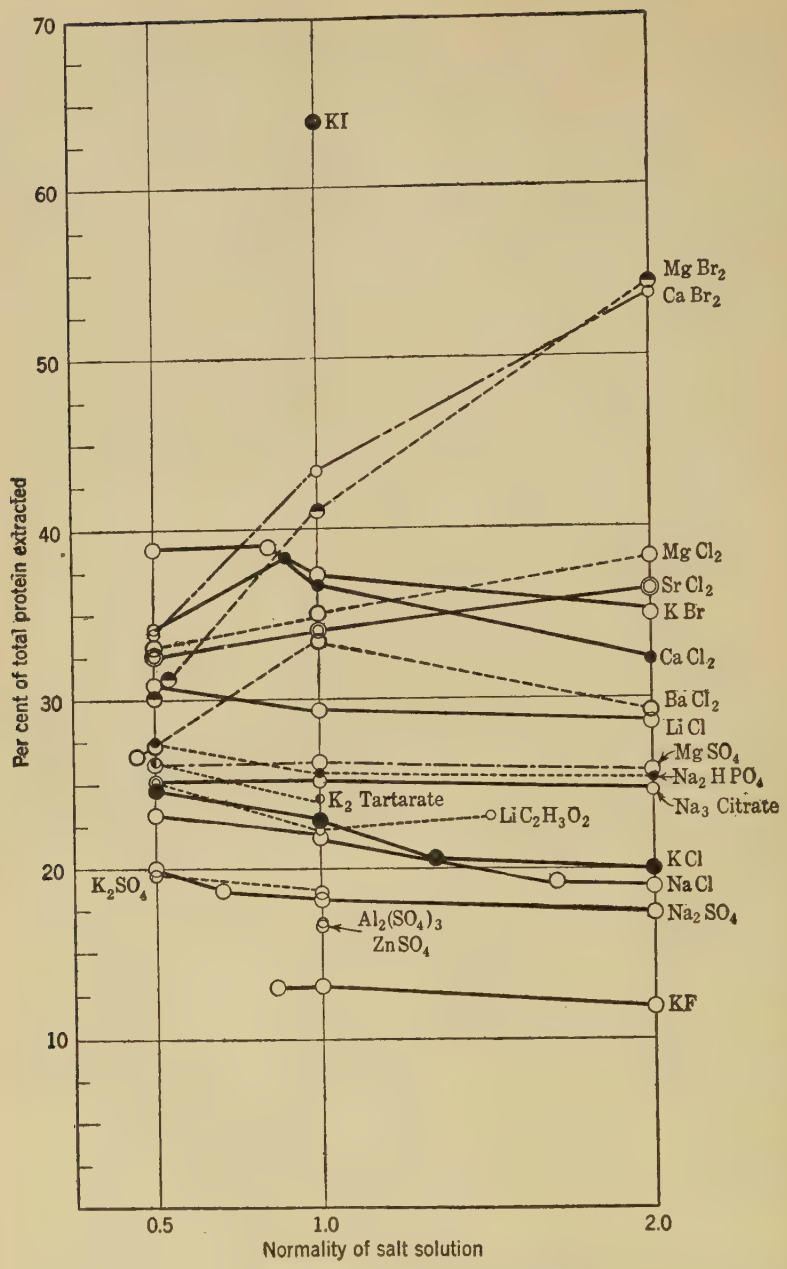


FIG. 103.—Showing the average percentage of the wheat flour proteins which is peptized by various salt solutions in various concentrations. (Data of Gortner, Hoffman, and Sinclair.)

TABLE XLVIII

Average Percentage of Total Protein* Extracted from (12) Wheat Flours by Various Concentrations of Salt Solutions

Salt	Concentration of Salt		
	0.5 N	1.0 N	2.0 N
Li acetate	25.01	22.32	
LiCl	30.08	29.32	28.60
NaCl	23.16	21.68	18.97
Na_2SO_4	20.00	18.20	17.30
Na_3 citrate	25.07	25.13	24.52
Na_2HPO_4†	27.44	25.62	25.32
KF	.....	13.07	11.93
KCl	24.62	22.77	20.01
KBr	38.77	37.22	34.91
KI	.....	63.89	
K_2SO_4	19.68	18.59	
K_2 tartrate	26.35	24.12	
K_2CrO_4	26.92	.....	
$MgCl_2$	33.01	34.99	38.35
$MgBr_2$	30.17	40.11	54.38
$MgSO_4$	26.18	26.35	25.69
$CaCl_2$	34.14	36.60	32.25
$CaBr_2$	33.90	43.27	53.68
$SrCl_2$	32.59	34.50	36.47
$BaCl_2$	27.29	33.42	29.24
$ZnSO_4$	.....	16.71	
$Al_2(SO_4)_3$	.....	16.90	

* Each figure represents the average of at least 24 separate nitrogen determinations.
† Considered as a divalent salt.

what, different results are obtained. This is to be expected if one considers that *the system in question is a colloid system and that solubility is synonymous with peptization.* On the other hand, it likewise means that the fractions which are isolated by a prescribed technic are not necessarily chemical entities but may represent only a peptized fraction.

We know far too little in regard to the physico-chemical factors underlying protein peptization to assign a cause for the variations noted in Table XLVIII. The cause is probably complex, involving the nature of amino acid linkages, secondary valence, polar groups, degree of hydration, specific ionic effects, electrokinetic forces, etc., and until at least some of these have been evaluated, it is useless to speculate as to the causes which are involved. On the other hand, the mere fact that one

can repeatedly isolate a given quantity of protein from biological material, using a rigidly standardized technic is no valid proof that the material which is isolated constitutes a chemical entity.

The term, globulin, is assigned to a group of proteins, separable from other protein fractions of tissues by a purely arbitrary procedure. The globulins have been separated into euglobulins and pseudoglobulins on an equally arbitrary basis. When a globulin changes its solubility and is transformed into a protean, such as myosin to myosan, it is classified as a " derived protein," although nothing is known of the chemical change which has come about, and although the protean has practically all of the properties of the class of simple proteins, known as glutelins. No one can say that the glutelins are not proteans which have undergone the globulin → protean transformation in nature.

The above discussion is inserted at this point not to confuse the student but rather to clarify his ideas in regard to protein classification. The author admittedly has no better classification to offer than the classifications which have already been presented. It is felt, however, that the research worker should recognize that solubility is nothing more nor less than peptization and that the proteins must be considered not alone as complex organic compounds but likewise as colloid micelles, subject to all of the varied reactions of a lyophilic system. Only under such conditions will the study of protein classification and reactions characteristic of protein systems be advanced.

CHAPTER XVI

CHARACTERISTIC PROPERTIES OF PROTEIN SYSTEMS

As already indicated, the reactions of protein systems must be those which are characteristic not only of the organic groups making up the protein molecule but also those which would be expected of lyophilic colloid systems. The literature in this field is so voluminous that the properties of only a few proteins can be mentioned and these very briefly and inadequately. It is hoped, however, that it will be possible to consider in a casual way some of the more important phenomena which are involved.

I. The Albumins.—Egg albumin and serum albumin are the two proteins of this class which have been most extensively investigated. We will confine our discussion almost entirely to a consideration of certain properties of egg albumin and egg albumin systems.

Both egg albumin and serum albumin are characterized by the fact that these proteins can be obtained in crystalline form. Accordingly, they must be regarded as being chemical entities. While the fact that the albumins could be obtained in crystalline form has been known for many years, a precise study of the crystallization process has been made by Sörensen[1] only recently.

Sörensen's technic involves the addition of one volume of neutral saturated ammonium sulfate solution to one volume of clear egg white. The mixed solution is filtered and saturated ammonium sulfate solution added to the filtrate to the point of incipient turbidity. To this solution there is then added 0.2 N sulfuric acid solution very slowly, with rapid stirring. The initial turbidity disappears, then a precipitate forms which dissolves on stirring, but the solution of the precipitate becomes more and more difficult with continued addition of sulfuric acid until finally a point is reached where all of the precipitate is dissolved, but no more would dissolve if additional sulfuric acid were added. At this stage, crystallization will begin within an hour or two and proceed for several days. The crystals may then be filtered by suction and washed with an

[1] Sörensen, S. P. L., Studies on the Proteins, *Compt. rend. trav. lab. Carlsberg*, Vol. XII, 372 pp. (1917).

ammonium sulfate solution, the concentration of ammonium sulfate being adjusted to the point where it will just fail to precipitate protein from the mother liquor. The crystals are then redissolved and recrys-

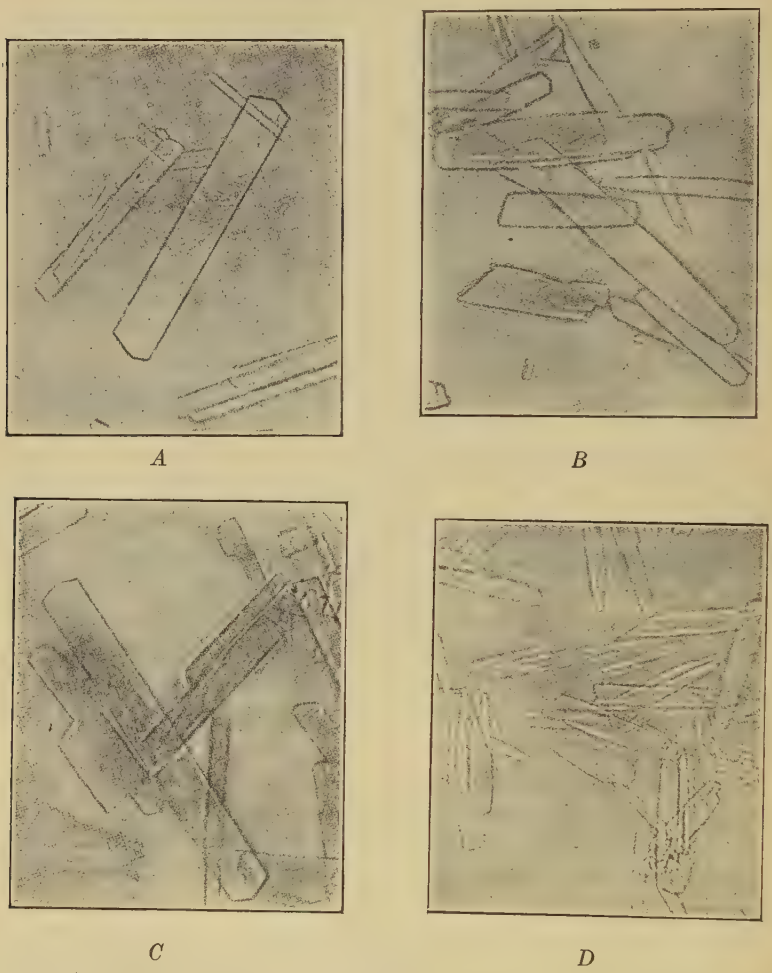


FIG. 104.—Crystals of egg albumin. (A) from a solution poor in protein, rich in ammonium sulfate, *p*H 5.0. (B) from a solution rich in protein, poor in ammonium sulfate, *p*H 4.8. (C) from a solution containing an excess of ammonia, *p*H 5.3. (D) from a solution containing an excess of sulfuric acid, *p*H 4.4. (After Sörensen.)

tallized by repeating the above process. Sörensen found that by this method three recrystallizations were sufficient to free the egg-albumin crystals from all the other proteins characteristic of egg white. The initial crystallization was best carried out at a final *p*H of 4.604, the

second recrystallization at a *p*H of 4.652, and the third recrystallization at a *p*H of 4.699. Figure 104 shows the form of the crystals of egg albumin which Sörensen obtained.

Sörensen studied the question as to the composition of these crystals and from an elaborate set of experiments found that they contained water of crystallization equivalent to 0.22 gram water per 1 gram water-free egg albumin.

In a study as to whether or not the crystals contained an excess of ammonia or of sulfuric acid derived from the ammonium sulfate, he found that only at a hydrogen ion concentration of 13×10^{-6} or a *p*H of 4.886 did the crystals contain no surplus of either ammonia or sulfuric acid. At a higher hydrogen ion concentration, an excess of sulfuric acid was present, and at a lower hydrogen ion concentration, an excess of ammonia. Figure 105 shows the form of the curves at varying hydrogen ion concentrations.

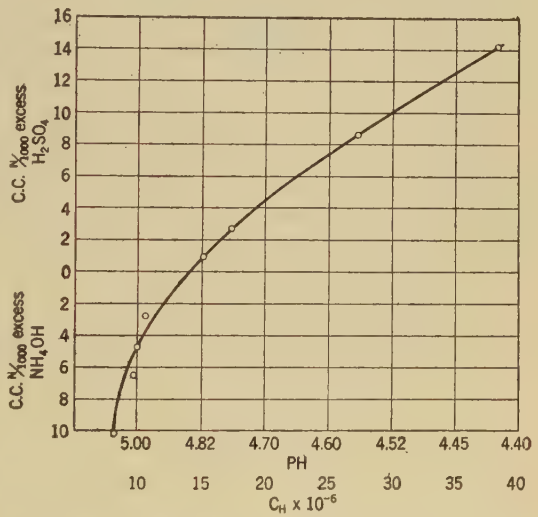


FIG. 105.—Showing the excess acid (H_2SO_4) or alkali (NH_4OH) per milligram equivalents of protein nitrogen present in crystalline egg albumin in equilibrium with solutions having different hydrogen ion concentrations. (Data of Sörensen.)

Sörensen further concludes that the crystals do not contain ammonium sulfate as an integral component. The best defined crystals, however, are obtained at a hydrogen ion concentration where a certain amount of sulfuric acid is present in the crystal, so that he regards the crystal as the crystal of a hydrous egg-albumin sulfate containing 0.22 gram water per gram of dry albumin and approximately one equivalent of sulfuric acid per 125 equivalents of protein nitrogen. Such crystals separate at a *p*H of 4.58 which appears to be the optimal *p*H for crystal formation.

In his study of the egg-albumin-ammonium-sulfate-water systems, Sörensen applied the phase rule of Gibbs to the crystallization process. He points out that other workers have attacked this problem but that in each instance they were working with egg albumin purified by precipi-

tation and not by repeated crystallization. Sörensen finds the Gibbs' phase rule to hold rigidly in an egg albumin-salt-water system, providing that the hydrogen ion is considered as one of the components, and he points out that the fact that previous workers failed to secure agreement with the phase rule was due to their regarding the system as a 3-component system, whereas in reality it is a 4-component system in which equilibrium can be reached only at a constant temperature, a constant concentration of ammonium sulfate, and a constant hydrogen ion concentration. When these three factors are held constant, the content of egg albumin in the mother liquor which is in equilibrium with the crystals will always be the same within experimental error. The state of equilibrium is therefore independent of the initial concentration of protein in the solution, so that from a concentrated albumin solution abundant crystals will form, while from a dilute solution only a small amount of crystals may be expected.

Inasmuch as he was working with a material having a very low diffusion coefficient, Sörensen found that equilibrium was very slowly reached, in most instances equilibrium not being reached in less than eight days and in some instances not until after thirteen days. Here again, he points out that previous workers had not waited sufficiently long for equilibrium to be attained.

Sörensen investigated certain other factors influencing crystallization and found, as might be expected, that the crystallization begins sooner and proceeds more rapidly as the concentration of ammonium sulfate is increased. When minimal amounts of ammonium sulfate were present, equilibrium was not reached even after thirteen days. The final equilibrium in all cases, however, was the same, irrespective of the concentration of ammonium sulfate, *i.e.*, the crystals in each instance had the same chemical composition.

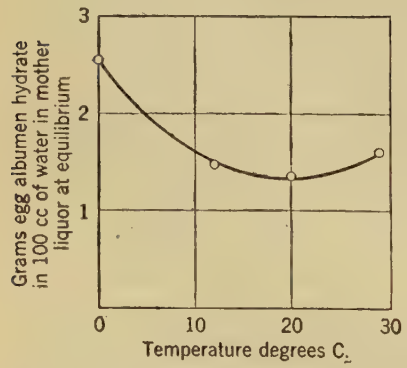


FIG. 106.—The effect of temperature on the crystallization equilibrium of egg albumin solutions. (Data of Sörensen.)

In a study of the effect of temperature on the equilibrium and on the velocity of crystallization, he found that the velocity of crystallization at 0° was lower than at 12° or 24°, and that the equilibrium at 0° was essentially different from that at 12° or 24°. He finds that between 12° and 29°, probably at approximately 20°, there is an optimum temperature at

which the smallest amount of egg albumin hydrate is present in the solution which is in a state of equilibrium with the crystals. Thus, a solution in equilibrium at 0° will produce additional crystals if raised to 29°, while a system which is in equilibrium at 29° will be displaced in the other direction by a transference to 0°, and a part of the crystals will dissolve. Sörensen's equilibrium-crystallization curve at various temperatures is shown in Fig. 106.

In a study of the influence of hydrogen ion concentration on the velocity of crystallization and the resulting equilibrium, Sörensen finds that within the range of *p*H 4.2 to 5.0 there exists an optimal *p*H concentration at which crystallization, other conditions being equal, will advance the farthest. His crystallization curves in relation to *p*H are shown in Fig. 107.

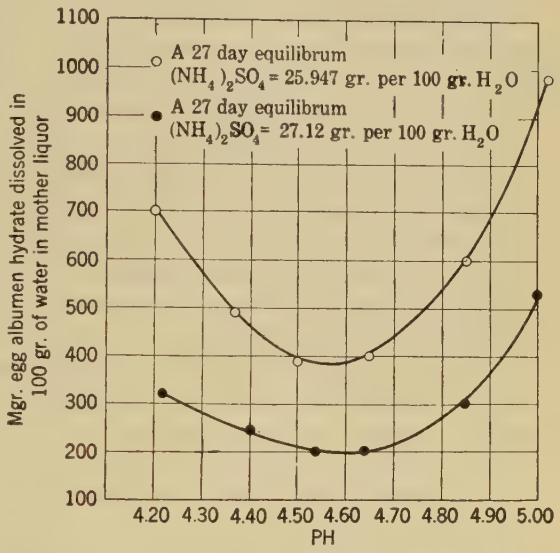


FIG. 107.—Showing the effect of hydrogen ion concentration on the "solubility" of crystalline egg albumin in ammonium sulfate solutions. (Data of Sörensen.)

The optimum point appears to be approximately *p*H 4.58. A decrease in hydrogen ion concentration from the optimum only causes much less albumin to crystallize out. (See sharp rise at decreased hydrogen ion concentration, Fig. 107.) The crystals which do form, however, are apparently normal in shape and character. An increase in hydrogen ion concentration causes only a slight increase in the solubility of the egg albumin hydrate crystals but does cause a decided alteration in both form and character of the crystals. The crystals contain much more sulfuric acid than normal. At a *p*H 5.22 to 5.30 the crystal form changes from small needle-shaped crystals to prisms.

The forms of the curves in Fig. 107 for the two different concentrations of ammonium sulfate indicate that it should be possible to find a higher concentration of ammonium sulfate at which an increase in *p*H would not affect the solubility of the crystals. Sörensen notes that this

is a possibility but did not carry out the necessary experiments to test this possibility.

Sörensen concludes his papers with a study of the osmotic pressure of egg albumin measured by the direct method. From a series of 18 different experiments, he finds that an egg-albumin solution of given composition will invariably show one and the same osmotic pressure, and as a result of his investigation, he reaches the conclusion that egg albumin has a molecular weight of about 34,000 and that it exists in solution in the form of simple molecules.

These observations of Sörensen have led numerous investigators to believe that the fact that an egg-albumin solution is monomolecular necessitates abandoning the viewpoint that egg-albumin solutions show colloidal properties. We have already pointed out that the colloidal realm is defined on the basis of the size of the micelle, irrespective of whether the micelle represents a single molecule or an aggregate of molecules. If the molecule of egg-albumin hydrate is sufficiently large to form a particle having a diameter greater than $1m\mu$, then that particle must possess surface forces which are characteristic of the lyophilic colloids. Ultrafiltration experiments indicate very clearly that the egg-albumin molecule lies well within the boundaries of the colloid realm. Consequently, while Sörensen has shown that egg-albumin solutions are molecularly dispersed solutions and that they obey the physico-chemical laws, such as the phase rule, nevertheless the author does not feel that we can abandon the colloid viewpoint in dealing with even egg-albumin systems.

Egg-albumin systems are the only protein systems which have been proven to be molecularly dispersed. Apparently egg albumin, with the molecular weight of 34,000 represents approximately the minimal molecular weight of the proteins. Albumin molecules will dialyze through a fairly porous collodion membrane. Lactalbumin, while less studied than egg albumin, likewise appears to be in a monomolecular state, inasmuch as it will also dialyze through a fairly porous collodion membrane.

Schutz and Zsigmondy[2] have utilized the gold number to characterize the various proteins of egg white. The crude egg white was found to have a gold number of 0.1 to 0.2. After a series of recrystallizations they obtained a gold number for the crystalline egg albumin, lying between 2.0 and 8.0, whereas the filtrates from the crystallized egg albumin contained proteins with a lower gold number than the crude egg white. They find the following gold numbers for the various protein fractions isolated from egg white: crystalline egg albumin 2.0 to 8.0, ovoglobulin 0.02 to 0.05 ovomucoid 0.04 to 0.08, uncrystallized " conal-

[2] Schutz, Fr. N., and Zsigmondy, R., Die Goldzahl und ihre Verwertbarkeit zur Charakterisierung von Eiweissstoffen, *Beitr. Chem. Physiol. Path.*, 3 : 137–160 (1902).

bumin" 0.03 to 0.06. They note that the presence of even a small quantity of the other egg proteins can be detected in the crystalline egg albumin by making use of the gold number technic.

As already noted, egg albumin contains a carbohydrate radical. Numerous investigators have studied the nature of this carbohydrate group. Osborne and Campbell[3] found that even the purest crystalline egg albumin yielded an osazone of a carbohydrate, 10 grams of the protein yielding 0.3681 gram of osazone. They note that this osazone had a melting point different from glucosazone, but in calculating the percentage of carbohydrate in the protein, they state that it is probably from 3 to 5 per cent of carbohydrate "calculated as glucose." Probably this phrase, "calculated as glucose," is responsible for the numerous statements in the literature that the carbohydrate in the egg albumin is glucosamine. The author has been unable to find any proof in the various papers which state that the carbohydrate is glucosamine. The fact that it is not glucosamine has been substantiated by the work of Fränkel and Jellinek[4] who have isolated from egg albumin a disaccharide composed of glucosamine and mannose, in which the union joining the two sugar radicals is through the amino group, inasmuch as the glucosamine-mannose compound does not react to yield nitrogen in the Van Slyke apparatus. Incidentally, they point out that this is the first instance in which mannose has been found in a material of animal origin.

II. The Globulins.—Proteins which have been designated as globulins have been prepared from a great variety of vegetable sources, as well as from muscle tissue, blood serum, and other biological fluids. As has already been indicated, these proteins have been characterized as being soluble in dilute salt solutions and insoluble in pure water. The general method of preparation is to extract the tissue with approximately 10 per cent sodium chloride solution and to dialyze the extract. The proteins which are precipitated will be regarded as globulins, whereas the albumins remain in solution. A modification of this method is to add to the extract an equal volume of saturated ammonium sulfate solution, the globulins precipitating in half-saturated ammonium sulfate, the albumins remaining in solution. The precipitated protein is then redissolved and reprecipitated by half saturation with ammonium sulfate, dialyzed free of ammonium sulfate, dried, and analyzed.

[3] Osborne, T. B., and Campbell, G. F., The Protein Constituents of Egg White, Twenty-Third Report of the Connecticut Experiment Station, pp. 348–375 (1900).

[4] Fränkel, S., and Jellinek, C., Über die sogenannte Kohlehydratgruppe im Eiweiss. (Darstellung der Glucosamino-Mannose), *Biochem. Z.*, 185 : 392–399 (1927).

The crude globulins so prepared have in a number of instances been fractionated by adding different amounts of ammonium sulfate to the solution and separating the various fractions which are precipitated at the various concentrations of ammonium sulfate. As already noted in the preceding chapter, the solubility of globulins in salt solutions is undoubtedly a peptization process. Accordingly, doubt exists in the mind of the author as to whether or not many of the globulins which have been reported are in reality chemical individuals.

No thoroughgoing study of the globulins, comparable to the work of Sörensen on egg albumin, has been reported. It seems highly probable that in the isolation of the globulins by " dissolving " them in neutral salt solution, we are dealing with nothing more nor less than a peptization process, such as is indicated in Fig. 108, where an increase in salt concentration throughout a certain range alters the surface forces in such a manner as to favor dispersion, following which a further increase in salt concentration causes the dehydration or the salting-out of the protein micelles. In this respect, the peptization of protein, as diagrammed in Fig. 108, would differ from the peptization of the silver halide, as diagrammed in Fig. 6, only in the amount of salt required to bring about maximal dispersion.

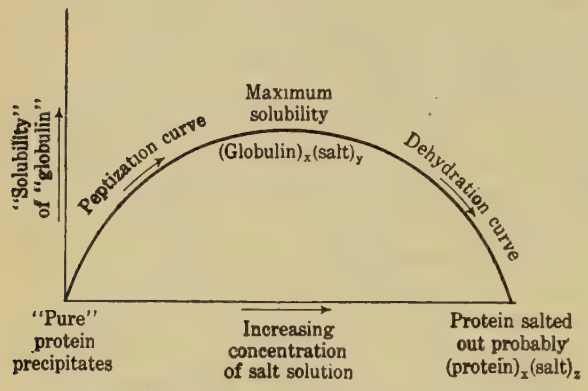


FIG. 108.—A diagrammatic representation of the interrelationships of certain of the factors concerned in protein "solubility."

Osborne and his coworkers[5] have summarized the literature in regard to the vegetable globulins. The globulins of animal origin which have been studied most extensively are those characteristic of blood serum. It is generally agreed, both from the chemical evidence and from the immunological evidence, that the globulin of the blood serum is identical with the globulin which occurs in milk. The albumins of blood serum and lactalbumin, however, are not identical.

Aside from their characteristic peptization behavior toward neutral

[5] Osborne, T. B., The Vegetable Proteins, Longmans, Green, and Company, New York (1909).

salt solutions, the chemical properties of the globulins are not greatly different from the chemical properties of many other proteins. Certain of the globulins can be obtained in crystalline form, but here again no exact study of the crystallization process has been made. Certain of the globulins, as already noted, pass into insoluble modifications, the proteans, on standing in contact with pure water or in the presence of hydrogen ions. Little is known in regard to the reason for this transformation which cannot be regarded as a characteristic property of all globulins, inasmuch as it is not shown by certain compounds classified as globulins.

III. The Prolamines.—The prolamines or proteins peptized by dilute alcohol, as already noted, are the characteristic proteins of the cereal grains. The name, prolamine, was given to this group of proteins by Osborne, inasmuch as at least certain of this group are proteins yielding on hydrolysis very large amounts of ammonia (acid amide nitrogen) and relatively large amounts of proline. The members of this group which have been most extensively studied are gliadin of wheat and rye, the zein of maize, and the hordein of barley. Osborne notes that all of the cereal grains have yielded alcohol-soluble proteins with the exception of rice.

Hoffman[6] reports the isolation of a small amount of an alcohol-soluble protein from rice. The chemical analysis of this protein, however, shows distinct differences from the general class of prolamines, and it may be that Hoffman's preparation was only a protein fraction which was peptized by the alcohol. No immunological study has been made to determine whether or not this fraction is distinct immunologically from the other rice proteins.

The literature of the prolamines, their chemical composition, and certain of their physico-chemical properties have been reviewed in a comparative study by Hoffman and Gortner[7] who point out that there is a great similarity in certain of the physico-chemical properties of the proteins of this group, whereas in other properties there is a gradation from gliadin which stands at one end of the series to the prolamines of sorghum and kafir which stand at the other end of the series. Thus, gliadin is peptized by cold 60 to 70 per cent alcohol, whereas the prolamines of kafir and sorghum are not peptized by the cold alcohol but

[6] Hoffman, W. F., An Alcohol-Soluble Protein Isolated from Polished Rice, *J. Biol. Chem.*, 66 : 501–504 (1925).

[7] Hoffman, W. F., and Gortner, R. A., Physico-Chemical Studies on Proteins. I. The Prolamines—Their Chemical Composition in Relation to Acid and Alkali Binding, Colloid Symposium Monograph, Vol. II, pp. 209–368, Chemical Catalog Company, Inc., New York (1925).

TABLE XLIX

The Nitrogen Distribution of the Various Prolamines as Determined by the Van Slyke Analysis, Expressed in Per Cent of Total Nitrogen

Protein	Ammonia N	Humin N		Basic N				N in filtrate from bases		Total N	Total Basic N
		Insoluble	Soluble	Arginine	Histidine	Cystine	Lysine	Amino	Non-Amino		
Gliadin	24.61	0.52	0.35	6.38	5.41	1.68	0.57	53.49	6.14	99.15	14.04
Speltin	24.18	0.49	0.56	6.58	4.45	1.02	3.07	53.49	5.50	99.34	15.12
Durumin	25.34	0.38	0.46	6.44	3.88	1.33	1.88	53.98	6.53	100.22	13.53
Dicoccumin	23.89	0.58	0.35	7.99	2.65	1.57	2.95	53.65	6.51	100.14	15.16
Monococcumin	24.19	0.89	0.64	7.20	5.05	1.13	2.30	53.05	4.50	99.05	15.68
Secalin	22.18	0.80	0.37	6.80	7.21	1.44	0.42	50.14	9.77	99.13	15.87
Sativin	22.20	0.70	0.55	8.93	2.31	1.74	1.20	54.60	5.86	98.08	14.18
Hordein	23.38	1.04	0.40	6.22	10.36	1.38	3.02	50.41	3.65	100.26	20.98
Zein	18.06	0.61	0.51	3.92	2.45	0.98	0.89	66.08	5.90	99.40	8.24
Teozein	18.99	0.69	0.50	3.90	3.58	0.80	2.57	64.44	3.62	99.09	10.85
Kafirin	20.76	0.69	0.66	3.92	1.71	1.23	2.48	68.85	0.32	100.62	9.34
Sorghumin	18.96	2.29	0.79	4.83	1.19	0.92	3.45	60.01	5.33	97.77	10.39

only by hot alcohol. Hoffman and Gortner divide the prolamines into two groups, a wheat group containing the prolamines from *Triticum vulgare, Triticum spelta, Triticum durum, Triticum dicoccum, Triticum monococcum,* and *Secale cereale,* and a corn group containing the prolamines from *Zea mays, Euchlaena mexicana, Andropogon sorghum,* and *Sorghum vulgare.* The proteins within these two groups have many similar properties, and Lewis and Wells[8] have shown that within the corn group and within the wheat group the prolamines show immunological relationships but that the two groups are distinct when tested by the biological method.

Mention has been made of the alcohol-soluble protein isolated from milk by Osborne and Wakeman.[9] While this protein is apparently a distinct protein and is peptized by alcohol, nevertheless it does not contain the large amount of ammonia nitrogen characteristic of most of the prolamines. It is doubtful, therefore, whether it should be classed as a true prolamine, inasmuch as its peptizability by alcohol appears to be the only property which relates it to this class of proteins. Table XLIX shows the nitrogen distribution of the various prolamines as determined by Hoffman and Gortner. These authors note that the prolamines are characterized by an almost complete absence of buffering action toward solutions of acid and alkali.

IV. The Glutelins.—These proteins, as noted, are characterized by not being peptized by neutral solvents but by being peptized by dilute acids and alkalies.

Larmour[10] has collected the literature in regard to these proteins and has added certain new glutelins to the known list. They are found in greatest amount in the cereal grains. Larmour notes that there is a progressive change in the chemical analyses of the glutelins which he studied, in regard to the ammonia nitrogen, arginine nitrogen, and total basic nitrogen content, ranging from glutenin from wheat at one end of the series to oryzenin from rice at the other end of the series, as shown in Table L.

In this class also the question arises as to whether the proteins which are isolated by a standard technic in reality represent chemical individuals. Tadokoro, Nakamura, and Watanabe[11, 12] have studied the

[8] Lewis, J. H., and Wells, H. G., The Immunological Properties of Alcohol-Soluble Vegetable Proteins. IX. The Biological Reactions of the Vegetable Proteins, *J. Biol. Chem.*, 66 : 37–48 (1925).

[9] Osborne, T. B., and Wakeman, A. J., Some New Constituents of Milk III. A New Protein, Soluble in Alcohol, *J. Biol. Chem.*, 33 : 243–251 (1918).

[10] Larmour, R. K., A Comparative Study of the Glutelins of the Cereal Grains, *J. Agr. Res.*, 35 : 1091–1120 (1927).

[11] Tadokoro, T., Nakamura, Y., and Watanabe, S., Differences of the Physico-

TABLE L

Values of Arginine Nitrogen of the Various Glutelins Arranged in Order of Magnitude, With Corresponding Values of Total Basic Nitrogen and Ammonia Nitrogen

Glutelin	Arginine N, Per Cent	Total Basic N, Per Cent	Ammonia N, Per Cent
Glutenin (Blish)	9.27	18.94	16.5
Zeanin	9.45	18.84	11.32
Glutenin (Larmour)	10.90	18.80	14.78
Duro-glutenin	11.00	21.20	13.25
Hordenin	11.08	20.25	11.38
Monococco-glutenin	11.86	20.16	10.78
Dicocco-glutenin	13.03	24.60	11.06
Spelta-glutenin	13.43	24.44	8.06
Secalenin (average)	13.77	25.69	9.24
Teozeanin	14.15	26.84	10.16
Avenin	15.48	24.37	11.87
Oryzenin	17.95	27.59	8.07

glutelins of rice varieties and subvarieties and point out that they find significant differences in the proteins isolated from the different varieties. Here again the question arises as to whether the proteins isolated represent chemical individuals or mixtures of various proteins which occur in different proportions in the different materials.

V. The Albuminoids.—As already noted, this is a heterogeneous class, generally resistant to chemical reagents and to peptic and tryptic enzymes, including such proteins as silk, wool, hair, skin, nails, horn, etc. Certain of these proteins appear to be made up of relatively few amino acids.

Raw silk is a mixture of two proteins, silk gelatin and silk fibroin, both being classed with the albuminoids. Silk gelatin is peptized by water under slight pressure, or by dilute alkaline solutions, and amounts to 15 to 20 per cent of the weight of the raw silk. Silk fibroin is characterized by its high content of three amino acids, glycine, alanine, and tyrosine, these three accounting for from 60 to 70 per cent of the weight of the silk fibroin. Silk, likewise, contains a relatively high percentage of serine (1 to 3 per cent has been actually isolated). Silk gelatin, in contrast to silk fibroin, is extremely low in its glycine content, somewhat

Chemical Properties of the Protein, Oryzanin, as Found in Glutinous and in Common Rice, *J. Coll. Agr. Hokkaido Imp. Univ.*, 14 : 129–169 (1925).

[12] Tadokoro, T., Nakamura, Y., and Watanabe, S., Physico-Chemical Studies on the Specificity of Proteins of Different Rice Varieties and Subvarieties, *J. Coll. Agr. Hokkaido Imp. Univ.*, 16 : 73–88 (1926).

higher in its serine content (5.4 to 6.6 per cent has been isolated), but the analyses of silk gelatin account for only 20 to 40 per cent of the amino acids which are present, so that, in general, little can be said in regard to its amino-acid content.

Certain of the albuminoids are of special interest. Thus, *spongin*, the skeletal protein of sponges and coral, contains no tyrosine but instead 3.5 di-iodo tyrosine or gorgonic acid. The iodine content of spongin ranges from 1 to 1.5 per cent. It is interesting to note that the ancient Greeks considered ground sponges to be specific for goiter. This view, along with many viewpoints of the ancients, was held up to ridicule until recent investigations have shown that certain forms of goiter are due to lack of iodine. Modern investigation has shown that the iodine present in 3.5 di-iodo tyrosine is effective in the prevention of goiter, and dried ground sponges have again been listed as an official remedy in the French Pharmacopœia.

Many of the keratins, notably human hair and wool, contain a very high percentage of cystine, human hair containing as high as 14 per cent of this amino acid. Accordingly, human hair is generally used as the protein for cystine isolation, although wool, which contains considerably less cystine, can be used if necessary.

Elastin, the principal protein of tendons, cartilage, bones, and connective tissue is noted for its high content of glycine and leucine, these two amino acids making up approximately half of the total weight of the protein.

Gelatin, obtained by extracting hides, bones, etc., with hot water is interesting because of the absence, or almost complete absence, of three amino acids, tryptophane, cystine, and tyrosine. A fourth amino acid, histidine, is present only in exceedingly small amounts. The proline and glycine contents are high, 7.7 and 6.5 per cent, respectively.

Gelatin is the only protein, insofar as the author is aware, which does not give the typical immunological reactions. It can accordingly be given intravenously without producing the deleterious effects which follow the intravenous injection of foreign proteins. During the World War, intravenous injections of gelatin were made in cases of excessive hemorrhage where considerable loss in blood volume had occurred, the gelatin sols replacing for a time the blood proteins which had been lost, thus maintaining an increased blood volume, and the corresponding higher blood pressure which was desirable. Later in the War the gelatin injections were replaced by injections of gum acacia which answered the same purpose. We have here an example of the importance of the lyophilic colloids in holding liquid in the blood vessels and maintaining normal blood volume. Bayliss has made the statement that by the use

of gelatin and later gum acacia in cases of excessive hemorrhage, at least 20,000 lives of wounded men were saved in the Allied Forces alone.

According to Osborne, albuminoids do not occur in the vegetable kingdom.

VI. The Histones.—These are basic proteins, characterized by a relatively high proportion of diamino acids and by the fact that at least in some instances they form salts with the strong mineral acids. Their nitrogen content ranges in the neighborhood of 17 to 20 per cent. They combine readily with nuclein or nucleic acid, and are the characteristic proteins of tissues rich in cells, such as glandular tissues. It has been suggested that the histones represent the union of a protamine with some other protein. The protein of hemoglobin is a histone combined with the non-protein radical, hemin.

VII. The Protamines.—The protamines, as already noted, are the characteristic proteins of ripe sperm cells and are apparently the proteins associated with nucleic acid in *chromatin*. The nitrogen content is high, ranging from 25 to 30 per cent of the weight of the protein. They are sulfur-free; so strongly alkaline that they will blue litmus paper. They spontaneously absorb carbon dioxide from the air, forming a more or less stable carbonate. They are easily soluble in water, insoluble in alcohol and ether, do not coagulate, and do not diffuse through parchment membrane. With mineral acids they form stable compounds, many of which are crystalline. Double salts of constant composition are formed with platinic chloride and auric chloride. They are not digested by pepsin but are hydrolyzed by trypsin and erepsin.

Salmin, the protamine from ripe salmon sperm, has been the most extensively studied. Apparently its composition is completely known. It has a formula, $C_{81}H_{155}N_{45}O_{18}$, a nitrogen content of 30.80 per cent, a molecular weight of 2045 or some multiple of this, an optical rotation, $[\alpha_D]$, of $^{-}81^{\circ}$, and the hydrolytic products contain 87.4 per cent of arginine, 11.0 per cent of proline, 7.8 per cent of serine, and 4.3 per cent of amino valerianic acid, which indicates the presence of 10 molecules of arginine, 2 molecules of serine, 2 molecules of proline, and 1 molecule of amino valerianic acid, in the salmin molecule.

Clupein, from herring sperm, has the same empirical formula as that of salmin. It contains 88 to 89 per cent of the total nitrogen in the form of arginine nitrogen, the other amino acids being alanine, serine, proline, and amino valerianic acid. It apparently differs from salmin only in the replacement of one molecule of serine by a molecule of alanine.

Sturin, from sturgeon sperm, contains all three of the diamino acids in the proportions of arginine 58.2 per cent, histidine 12.9 per cent, lysine 12.9 per cent, the three totaling 84 per cent of the protein mole-

cule. The remaining amino acids are apparently exclusively leucine and alanine.

Scombrin, from mackerel sperm, is probably the simplest protamine, apparently containing only three amino acids, arginine 88.8 per cent of the total nitrogen, proline 3.8 per cent of the total nitrogen, the remainder being alanine, although there is a possibility that some other amino acid may be present in the alanine fraction.

Kossel and his students have done the most extensive work in the field of the protamines. He has presented in two papers[13, 14] summaries of certain of his investigations.

Thompson[15] has studied the physiological action of protamines. When they are injected intravenously, they cause a rapid fall in blood pressure and decreased respiration, death ensuing at even low dosages. The fatal dose of clupein, injected intravenously, lies between 0.15 and 0.18 gram for a 10 kilogram dog. The lethal dosage of sturin lies between 0.20 and 0.25 gram for a dog of similar weight. In the case of a non-lethal dose, the initial reaction is over after a thirty-minute period, but a second dose causes the same symptoms, indicating that there is conferred no appreciable degree of immunity. Insofar as the blood picture is concerned, the intravenous injection causes a marked delay in blood-coagulation time, the coagulation being delayed for as much as thirty-six hours. The same effect is produced on blood coagulation *in vitro*. Leucocytes are rapidly destroyed by the intravenous injection of protamines. A sublethal injection of sturin reduced the leucocyte count from a normal count of 28,281 to 1875 after a single injection and to 781 after a second injection. The diamino acids alone did not produce the above physiological symptoms, nor did a mixture of all of the hydrolytic products of the protamine. Thompson accordingly concludes that the toxic action is due to the specific groupings in the molecule.

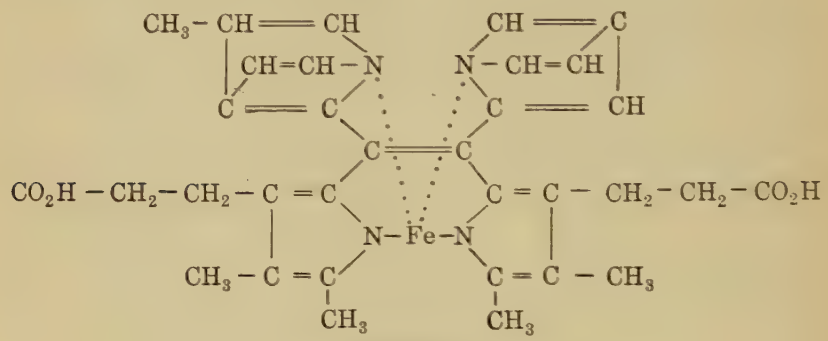
VIII. The Chromoproteins.—As already noted, the chromoproteins include the *hemoglobins* characteristic of the red blood cells of the mammals. In the case of hemoglobin, the associated radical, hemin or hemochromogen, has the approximate formula, $C_{34}H_{30}N_4O_4Fe$, united with a histone. The oxygen-carrying capacity of the blood is associated with the pigmented radical and is probably due to the presence of iron in this radical. The hemoglobins contain from 0.33 to 0.51 per cent of

[13] Kossel, A., Einige Bemerkungen über die Bildung der Protamine im Tierkörper, *Z. physiol. Chem.* 44 : 347–352 (1905).

[14] Kossel, A., and Pringle, H., Über Protamine und Histone, *Z. physiol. Chem.*, 49 : 301–321 (1906). *Cf.* also "The Protamines and Histones," by A. Kossel. Translated by W. V. Thorpe. Longmans, Green and Co., New York (1928).

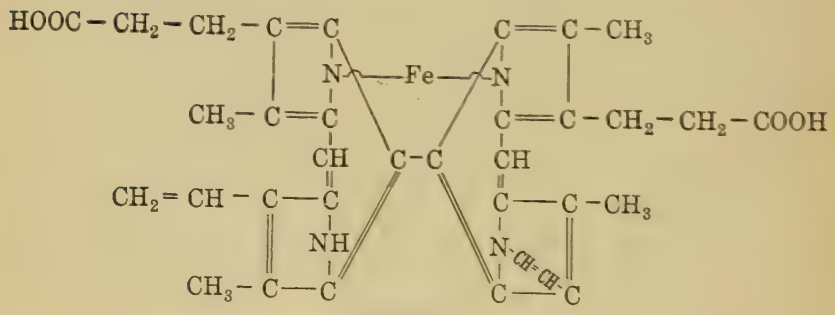
[15] Thompson, W. H., Die physiologische Wirkung der Protamine und ihrer Spaltungsprodukte, *Z. physiol. Chem.*, 29 : 1–19 (1900).

iron, according to the species, and have a minimum molecular weight, based on the iron content, of from 16,000 to 17,000. The radical which is associated with the histone is apparently built up from a series of pyrrole rings linked together by carbon chains containing double bonds as shown in the following formulae:



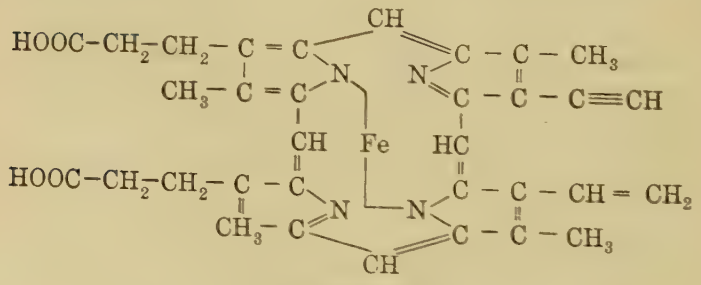
$C_{33}H_{32}O_4N_4Fe$

Willstätter's formula of hemin (*Z. physiol. Chem.*, 87 : 434, 1913)



$C_{34}H_{30}N_4O_4Fe$

Hans Fischer's formula of hemin (*Z. physiol. Chem.*, 142 : 160, 1925)



$C_{34}H_{30}N_4O_4Fe$

Küster's formula of hemin (*Z. physiol. Chem.*, 163 : 282, 1927)

If one compares these formulae with the suggested formula of chlorophyll (*vide infra*), it will be noted that there is a striking similarity, indicating that the most important pigment of the plant kingdom and the most important pigment of the higher animals have much in common and may be actually modifications of the same structure.

Warburg has within recent years been paying particular attention to the role of iron in the process of oxidation. He[16] has summarized his work in this field and reported new findings. This paper of Warburg is very suggestive, and if his viewpoint is correct, marks a striking advance in the study of vital phenomena. He notes that *hemin*, the nucleus of hemoglobin, is present not only in the form of hemoglobin in animal cells and tissues but that *it is likewise present in all plant and animal cells*, including the bacteria and the yeasts, the only difference being that in the red blood cells it is combined with the protein, globin, whereas in other cells it may exist either free, or combined with other compounds.

Apparently the combination between hemin and globin to form hemoglobin is not a direct union of carbon with carbon, such as characterizes many organic compounds, but is more like that of an acid with a base, inasmuch as Hill and Holden[17] have succeeded in recombining hemin with the protein, globin, thus effecting its resynthesis at least insofar as the union of these two radicals is concerned. Other bases, much more simple than globin, can be combined with hemin; for example, the bases, pyridine and nicotine, form hemopyridine and hemonicotine, respectively. Both these, and similar compounds, have properties which are somewhat different from hemoglobin, but as Warburg notes, in many reactions their properties, while modified, are the properties of the hemin nucleus.

The combining capacity of hemoglobin is due to a combination of hemin with molecular oxygen, in which one atom of iron in the hemin unites with one molecule of oxygen. The only function of the hemoglobin in the blood stream is to carry this molecule of oxygen from the lungs to the various cells and tissues where the oxygen pressure is lower, under which condition the oxygen is released from the hemoglobin and diffuses through the capillary walls into the tissue cells where it is utilized for respiration. Warburg states that hemoglobin itself has no catalytic action in stimulating respiration.

[16] Warburg, O., The Chemical Constitution of Respiration Ferment, *Science*, 68 : 437–443 (1928), translated from the German, *Naturwissenschaften*, 16 : 345–350 (1928).

[17] Hill, R., and Holden, H. F., The Preparation and Some Properties of the Globin of Oxyhaemoglobin, *Biochem. J.*, 20 : 1326–1339 (1926).

It is well known that carbon monoxide acts as a toxic gas because of the formation of carbon-monoxide hemoglobin in which the carbon monoxide is more firmly held on the iron atom than is molecular oxygen. Warburg points out that in experiments conducted by Haldane where mice were exposed to an atmosphere of carbon monoxide, the carbon monoxide displaced the oxygen in hemoglobin and the mice died for lack of oxygen. When, however, such mice before death were placed in an atmosphere of higher oxygen content, the total hemoglobin still remained combined with carbon monoxide but the dissolved oxygen in the blood plasma increased ten-fold. In this case, the mice did not die because the oxygen dissolved in the blood plasma was sufficient to supply the tissues for the process of respiration. Warburg points out that this experiment indicates that carbon monoxide does not affect the respiration of the cells even when the hemoglobin is completely saturated with carbon monoxide.

Warburg then proceeds to the theory that the enzyme which is responsible for the respiration process is hemin or a hemin derivative. He notes that the equilibrium,

$$\text{oxyhemoglobin} + \text{carbon monoxide} \rightleftharpoons \text{hemoglobin-carbon monoxide} + \text{oxygen}$$

is shifted to the left when the system is exposed to light. In other words, the hemoglobin is regenerated when carbon-monoxide-hemoglobin is illuminated. He finds a similar effect of carbon monoxide upon respiration when the experiments are conducted in the dark and in the light. In the dark the respiration of cells is inhibited by carbon monoxide, the inhibition diminishing and disappearing rapidly when cells are illuminated. He notes that the intensity of light required to effect this photo-chemical change, insofar as the respiration enzyme is concerned, is only one ten-thousandth of the intensity of sunlight.

Warburg notes that the respiration enzyme has the three characteristic properties of hemoglobin. It reacts reversibly with carbon monoxide and oxygen; the carbon monoxide and oxygen show the same distribution coefficient,

$$\frac{HbO_2}{HbCO} \cdot \frac{CO}{O_2} = K$$

and the combination with carbon monoxide is sensitive to light. The difference between hemoglobin and the respiration enzyme is noted to be that the respiration enzyme acts as a catalyst, whereas hemoglobin does not. Accordingly, Warburg believes that the respiration enzyme approximates the properties of free hemin more closely than the prop-

erties of hemoglobin. He notes that free hemin catalytically oxidizes cysteine in aqueous solution to cystine, but that this catalysis is inhibited by carbon monoxide. Here again the system is only moderately sensitive to light. If, however, the hemin is combined with pyridine or nicotine, very powerful catalysts were obtained capable of transferring 2000 molecules of oxygen per molecule of hemonicotine or hemopyridine to the cysteine, and the carbon-monoxide compounds of these hemin derivatives had the same remarkable reaction toward light, the carbon monoxide being liberated by light of one ten-thousandth of the intensity of sunlight.

Warburg then studied the effect of light of various wave lengths on cell respiration in the presence of certain amounts of carbon monoxide

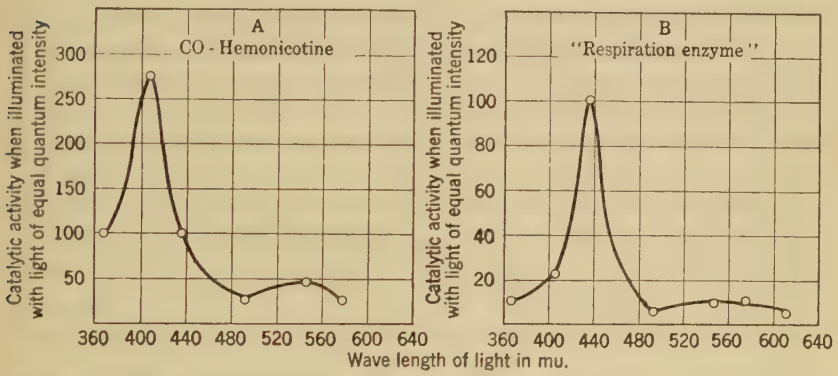


Fig. 109.—Showing the similarity in absorption spectra of carbon monoxide hemonicotine and the respiration enzyme (Curve *B*), as measured by studying their catalytic activity when illuminated with light of pure wave length and equal quanta intensity. (Data of Warburg.)

and secured what are essentially absorption spectrum curves, as measured with a photometer, for carbon-monoxide hemonicotine and his hypothetical carbon-monoxide respiration enzyme, using the respiration of living cells in the latter instance. *He finds the two absorption curves to be essentially identical*, the only difference being that the entire curve is shifted approximately $20m\mu$ toward the red end of the spectrum, as shown in Fig. 109. He notes that curve (*B*), showing the characteristic shift toward the red end of the spectrum, is characteristic of the spectrum of a solid compound, rather than of a dissolved compound, that when an absorption spectrum is taken of material in the solid state, there is regularly a shift of approximately $20m\mu$ toward the red end of the spectrum as compared to the curve of the material in solution He accordingly suggests that *the respiratory enzyme is hemin combined with some as yet*

unknown base and that it is not in solution in the cell but is imbedded in (adsorbed upon (?)) the surfaces of the solid cellular components.

If these observations of Warburg are shown to hold in the light of further investigation, it would appear that the nature and chemical formula of the most vital enzyme has been elucidated.

The blood of the cephalopods, such as the squid and octopus, and of the crustacea, such as lobsters, oysters, mollusks, etc., does not contain hemoglobulin but rather a chromoprotein known as *hemocyanin*, in which the iron is replaced by *copper*, the copper content ranging from 0.33 to 0.38 per cent. The color change of oxidized to reduced hemocyanin is from light blue to colorless. Relatively little is known in regard to the nature of either the associated chromogen or of the protein itself. Alsberg and Clark[18] report a Van Slyke analysis of the hemocyanin of the crab (*Limulus polyphemus*), in which they find 31.3 per cent of the total nitrogen to be present in the form of arginine, histidine, and lysine.

Stedman and Stedman[19] have recently made certain physico-chemical studies on hemocyanin, but much more chemical work is needed on these respiratory pigments before it can be definitely ascertained whether or not they are closely allied to hemoglobin in their chemical constitution. Incidentally, the recent announcement of Hart, Steenbock, Waddell, and Elvehjem[20] that certain types of anemia are benefited by the inclusion of small amounts of copper in the diet, suggests the possibility that mammals have not completely lost the vestiges of some vital mechanism associated with the formation of hemocyanin, even though iron has replaced copper in their principal respiratory pigment.

Similarly the observations of Griffith[21] show that the respiratory pigment in the tropical mussel, *Pinna squamosa*, contains neither iron nor copper, but rather *manganese* to the extent of 0.81 per cent. He has named this chromoprotein, *pinnaglobin*, although he does not give many chemical data by which it can be characterized. He notes that the

[18] Alsberg, C. L., and Clark, E. D., The Haemocyanin of Limulus Polyphemus, *J. Biol. Chem.*, 8 : 1–8 (1910).

[19] Stedman, Ellen, and Stedman, Edgar, Haemocyanin. Pt. I. The Dissociation Curves of the Oxyhaemocyanin in the Blood of Some Decapod Crustacea, *Biochem. J.*, 19 : 544–551 (1925); Pt. II. The Influence of Hydrogen Ion Concentration on the Dissociation Curve of the Oxyhaemocyanin from the Blood of the Common Lobster (*Homarus vulgaris*), *Biochem. J.*, 20 : 938–948 (1926); Pt. III. The Influence of Hydrogen Ion Concentration on the Dissociation Curve of the Oxyhaemocyanin from the Blood of the Edible Crab (*Cancer Pagurus*), *Biochem. J.*, 20 : 949–956 (1926).

[20] Hart, E. B., Steenbock, H., Waddell, J., and Elvehjem, C. A., Iron in Nutrition. VII. Copper as a Supplement to Iron for Hemoglobin Building in the Rat, *J. Biol. Chem.*, 77 : 797–812 (1928).

[21] Griffith, A. B., Sur la composition de la pinnaglobine; une nouvelle globuline, *Compt. rend.*, 114 : 840–842 (1892).

change from the oxidized to the reduced condition produces a color change from brown to colorless. Here again the observation of Titus and Cave[22] that manganese facilitates hemoglobin formation in the higher animals may indicate a previous condition where manganese was an essential constituent of a vital pigment.

As already noted, certain sea algae contain the chromoproteins, *phycoerythrin* and *phycocyan*. These have been studied by a number of workers of whom only two or three will be cited. The first extensive study is that of Kylin,[23] who prepared these pigments in crystalline form, identified them as of protein nature, studied their behavior toward various reagents, including enzymes, acids, and alkalies, and their reactions toward heat and light.

In a later study, Kitasato[24] isolated a reddish-violet, water-soluble pigment to which he gives the formula, $C_{48}H_{120}O_{18}N_{12}$, from phycoerythrin, which is further hydrolyzed by acids to yield a blue pigment, insoluble in water, soluble in organic solvents, and having a composition, $C_{30}H_{54}O_{12}N_6 \cdot 1.5\ H_2O$. He notes that phycocyan yields, on peptic digestion, the final blue pigment directly, the intermediate compound not being obtained.

Svedberg and Lewis[25] have studied the light absorption of these proteins and their minimal particle weight as determined in the ultracentrifuge. They find an average "molecular weight" of 101,800 for phycocyan, and 206,000 for phycoerythrin, indicating that they have a very complex constitution. The chemical nature of the colored group still remains for future investigation.

Gortner,[26, 27] in a study designed primarily as an investigation of the chemical nature of melanin, isolated from black wool by treatment with extremely dilute alkalies a material of protein nature which was intensely black but which on acid hydrolysis yielded about 90 per cent of its weight in amino acids. This material he regards as a conjugated protein in which melanin is the associated chromogen. Inasmuch as no similar compound could be isolated from non-pigmented wool, and inas-

[22] Titus, R. W., and Cave, H. W., Manganese as a Factor in Hemoglobin Building, *Science*, 68 : 410 (1928).

[23] Kylin, H., Über Phykoerythrin und Phykocyan bei Ceramium rubrum (Huds.) Ag., *Z. physiol. Chem.*, 69 : 169–239 (1910).

[24] Kitasato, Z., Biochemische Studien über Phycoerythrin und Phycocyan, *Acta Phytochimica*, 2 : 75–98 (1926).

[25] Svedberg, The, and Lewis, N. B., The Molecular Weights of Phycoerythrin and of Phycocyan, *J. Am. Chem. Soc.*, 50 : 525–536 (1928).

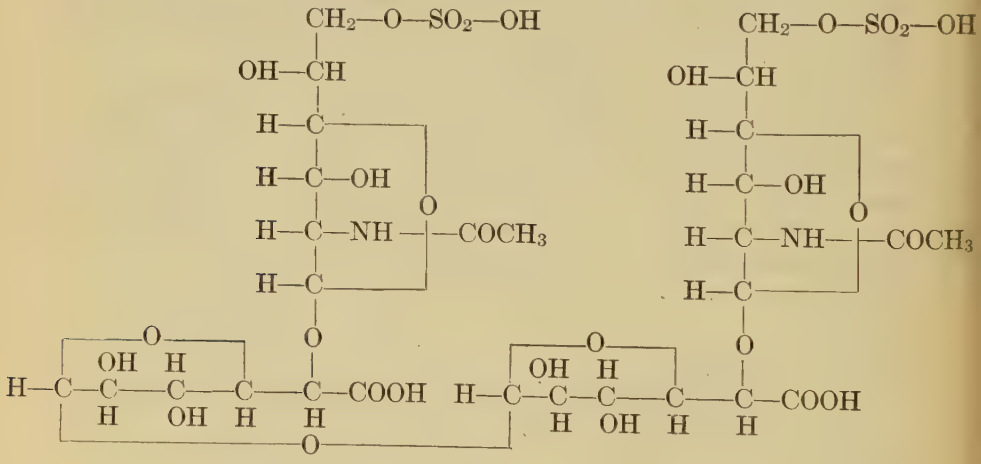
[26] Gortner, R. A., Studies on Melanin, I. Methods of Isolation, The Effect of Alkali on Melanin, *J. Biol. Chem.*, 8 : 341–363 (1910).

[27] Gortner, R. A., On Melanin, *Biochem. Bull.*, 1 : 207–215 (1911).

much as certain other pigmented structures, such as numan hair, black rabbit fur, and black feathers did not yield a similar product, it seems reasonable to believe that the pigment of black wool exists in the form of *melano-proteins* rather than in the form of melanin which is not associated with the protein molecule. Here again, however, further work is necessary before this group of compounds can be definitely characterized.

IX. The Glycoproteins.—We have already noted that egg albumin contains a carbohydrate radical. The amount of carbohydrate which is present, however, is extremely small in comparison with the amount of carbohydrate in the group known as the mucins and the mucoids. The mucin contained in the submaxillary glands has been reported to contain 23.5 per cent of carbohydrate, salivary mucin to contain as high as 36.9 per cent of carbohydrate, and tracheal mucin from 34 to 37 per cent of carbohydrate. The group which is associated with the protein has been studied particularly by Levene[28] and his coworkers. Levene has prepared a summary of their investigations. He distinguishes two types of groups associated with the protein, *i.e.*, chondroitin sulfuric acid and mucoitin sulfuric acid.

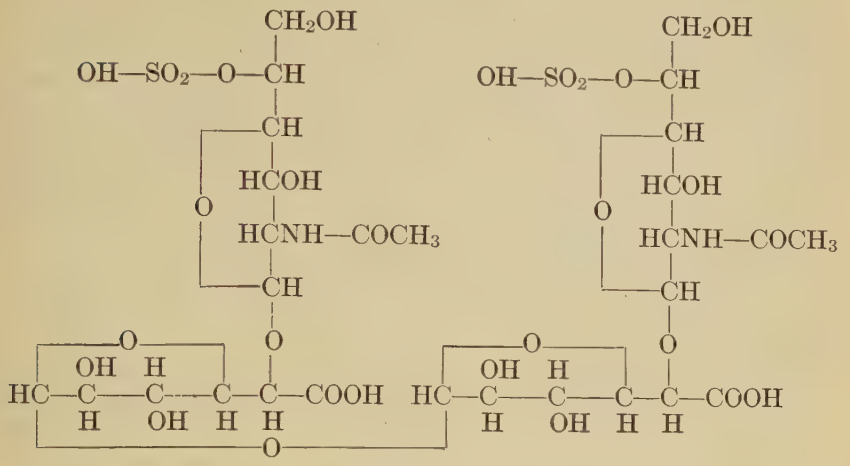
Chondroitin sulfuric acid on hydrolysis yields chondrosamic, glucuronic, acetic, and sulfuric acids in equimolecular proportion. Levene, accordingly, presents the following graphic formula as representing the structure of chondroitin sulfuric acid:



Similarly, the decomposition products of mucoitin sulfuric acid have been identified as mucosin, a disaccharide composed of glucuronic acid

[28] Levene, P. A., Hexosamines, Their Derivatives, and Mucins and Mucoids, The Rockefeller Institute for Medical Research Monograph No. 18, 104 pp. (1922).

and chitosamine, acetic acid, and sulfuric acid. Levene presents the following structural diagram as the probable formula:



When the sulfuric acid radical is removed from the chondroitin sulfuric acid or from the mucoitin sulfuric acid, substances are formed which are non-reducing, relatively inert chemically, and in many respects resemble gum acacia (gum arabic) in properties. These compounds are highly hydrophilic and probably account for the very hydrophilic properties of the mucins and mucoids with which they are associated in the conjugated proteins.

X. The Phosphoproteins.—*Casein* of milk and *vitellin* of egg yolk are the two most important phosphoproteins. Casein, because of its ease of preparation, has been most extensively worked with. The chemistry of casein has been adequately discussed by Sutermeister and co-authors[29] in a recent publication, and in the recent American Chemical Society Monograph dealing with Dairy Science.[30] Consequently we will not devote any considerable amount of space to a discussion of this protein.

It is of interest to note that casein is the characteristic protein of the milk of all species of mammals, even the egg-laying monotreme or spiny ant-eater, *Echidna aculeata multi-aculeata*, secreting in its mammary

[29] Sutermeister, Edwin, Casein and Its Industrial Applications, American Chemical Society Monograph Series, Chemical Catalog Company, Inc., New York (1927). Note in particular Chapter I.

[30] Associates of Lore A. Rogers, Fundamentals of Dairy Science, American Chemical Society Monograph Series, Chemical Catalog Company, Inc., New York, (1928).

glands a protein having the characteristics of casein.[31] Similarly, vitellin appears to be the characteristic protein of egg yolk, although the various vitellins have not been investigated as extensively as have the caseins.

The caseins are acted upon by a special enzyme, rennin, which converts them into another protein, *paracasein*, having somewhat different properties. Palmer and Richardson[32] have reported on the colloidal chemistry of the rennet coagulation process and have studied the effects of acids and alkalies on paracasein, using technic similar to that used by Hoffman and Gortner[33, 34] in their studies of casein. Palmer and Richardson find that the action of rennet is to increase the number of free acidic groups in paracasein, so that it binds a greater amount of alkali per gram than does casein. The mechanism by which this change is produced is still uncertain.

XI. The Nucleoproteins.—The nucleoproteins consist of a protein group, usually a *histone* or *protamine*, combined with *nucleic acids*. The nucleic acids have been extensively studied. Jones[35] and Levene[36] have been particularly interested in this field during recent years.

The nucleic acids which have been studied have been derived mainly from either the thymus gland or from yeast, the former being taken as a representative of the nucleic acids of animal origin, the latter of plant origin. On hydrolysis all nucleic acids yield phosphoric acid, a carbohydrate, two pyrimidine bases, and two purine bases. For a long time the statement was generally accepted that plant nucleic acids were all alike but that they differed from animal nucleic acids which were likewise all alike, the difference being that plant nucleic acids contained a pentose, whereas the carbohydrate of the animal nucleic acids was a hexose, and also that one pyrimidine base, uracil, occurred only in the plant nucleic

[31] Marston, H. R., The Milk of the Monotreme—*Echidna aculeata multi-aculeata Australian J. Exp. Biol. and Med. Sci.*, 3 : 217–220 (1926).

[32] Palmer, L. S., and Richardson, G. A., The Colloid Chemistry of Rennet Coagulation, Colloid Symposium Monograph, Vol. III, pp. 112–134, Chemical Catalog Company, Inc., New York (1925).

[33] Hoffman, W. F., and Gortner, R. A., Physico-Chemical Studies on Proteins, I. The Prolamines—Their Chemical Composition in Relation to Acid and Alkal. Binding, Colloid Symposium Monograph, Vol. II, pp. 209–368 (1925).

[34] Hoffman, W. F., and Gortner, R. A., Physico-Chemical Studies on Proteins.i II. Alkali Binding. A Comparison of the Electrometric Titration of Proteins and of Phosphoric Acid with Sodium and Calcium Hydroxides, *J. Phys. Chem.*, 29 : 769–781 (1925).

[35] Jones, W., Nucleic Acids, Their Chemical Properties and Physiological Conduct, Longmans, Green and Company, New York (1914).

[36] Levene, P. A., Numerous papers in the Journal of Biological Chemistry.

acids, whereas another, thymine, occurred only in animal nucleic acids. Recent investigations have shown, however, that the above statements are not necessarily true.

Levene has identified a pentose, *d*-ribose, in animal nucleic acids, and Jones and Perkins[37] have reported that uracil is probably derived from cystosine by secondary reactions in the method of isolation. This, however, is disputed by Levene.[38] Johnson and Coghill,[39] in addition, report a new pyrimidine, 5-methyl-cytosine in the nucleic acid of the tubercle bacillus. Johnson earlier[40] reported thymine in the nucleic acid of the tubercle bacillus, so that it would appear that the early distinction between plant and animal nucleic acids is pretty well broken down.

The evidence for a hexose in certain nucleic acids is based not upon the isolation of the sugar itself, but rather upon the formation of levulinic acid (γ-ketovalerianic acid, $CH_3COCH_2CH_2COOH$) when the nucleic acid is hydrolyzed. Levulinic acid is a characteristic decomposition product of hexoses.

The pentose associated with nucleic acid was identified by Levene and Jacobs in a series of papers during the years 1908 to 1911. Rather interestingly this pentose was not known to occur in nature until it was discovered as a decomposition product of yeast nucleic acid. Fischer, however, had synthesized it in 1901, during his studies on the carbohydrates. We have thus an example of a synthetic product prepared first in a chemical laboratory, which after a number of years was found to be identical with one of the most important, if not the most important, carbohydrate occurring in nature.

Recently a new sugar containing sulfur has been isolated from yeast, and there is certain evidence that it may be combined in a nucleic acid. This point will be returned to later.

Forbes and Keith[41] have given a scheme for the decomposition of nucleic acid on acid hydrolysis:

[37] Jones, W., and Perkins, M. E., The Nitrogenous Groups of Plant Nucleic Acid, *J. Biol. Chem.*, 62 : 557–564 (1925).

[38] Levene, P. A., On the Nitrogenous Components of Yeast Nucleic Acid, *J. Biol. Chem.*, 67 : 325–327 (1926).

[39] Johnson, T. B., and Coghill, R. D., Researches on Pyrimidines. CIII. The Discovery of 5-Methyl-Cytosine in Tuberculinic Acid, the Nucleic Acid of the Tubercle Bacillus, *J. Am. Chem. Soc.*, 47 : 2838–2844 (1925).

[40] Johnson, T. B., and Brown, E. B., The Pyrimidines Contained in Tuberculinic Acid. The Nucleic Acid of Tubercle Bacilli, *J. Biol. Chem.*, 54 : 731–737 (1922).

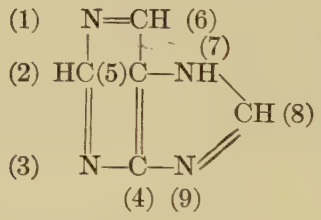
[41] Forbes, E. B., and Keith, M. H., A Review of the Literature of Phosphorus Compounds in Animal Metabolism, Ohio Agr. Exp. Sta. Tech. Series, Bull. No. 5, (1914).

H_3PO_4

Carbohydrates { Pentoses—*d-ribose*
Hexoses —levulinic acid

Bases { Purines { guanine, adenine } amino purines
xanthine, hypoxanthine } oxy purines
Pyrimidines { cytosine, thymine, uracil

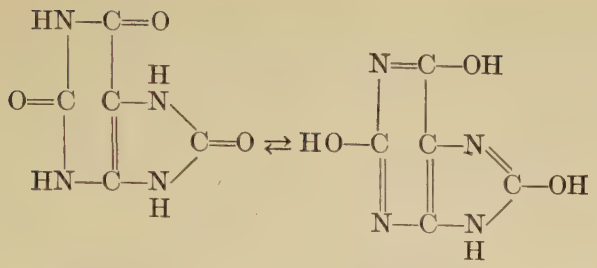
As a matter of fact, it is fairly well established that guanine and adenine are the only naturally-occurring purines in the nucleic acids and that xanthine and hypoxanthine are derived from these by the action of acids or enzymes. Purine, the mother substance of the purine bases, does not occur in nature but has been synthesized and has the following structure, the ring being numbered, as shown in the structural diagram:



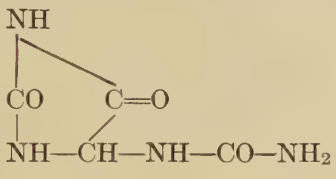
The various derivatives with which we are concerned are as follows:

Guanine = 2-amino-6-oxy-purine.
Adenine = 6-amino-purine.
Guanine + enzyme (guanase) = 2.6-dioxy-purine = *xanthine.*
Adenine + enzyme (adenase) = 6-oxy-purine = *hypoxanthine.*
Hypoxanthine by oxidation goes to xanthine.
Xanthine + oxygen + enzyme = *uric acid*, 2.6.8-trioxy-purine.

The oxygenated purine or pyrimidine compounds undergo keto $\rightleftharpoons$ enol isomerism and accordingly may exist in either the enol form or the keto form, or as an equilibrium mixture of the two forms:



Uric acid is the end product of purine metabolism in man, but in the lower animals it is further oxidized to allantoin:



which is further oxidized to urea,

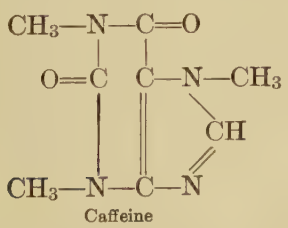
$$NH_2—CO—NH_2$$

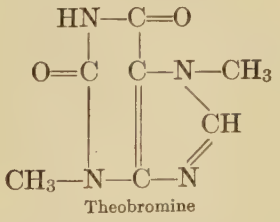
or

(Werner's formula)

The enzymes, adenase and guanase, are *deaminases*, the further oxidation to uric acid being produced by *oxidases*.

Besides the purines noted above, there are two others which are important from a phytochemical standpoint. These are *caffeine*, 1.3.7-trimethyl-xanthine, and *theobromine*, 3.7-dimethyl-xanthine:

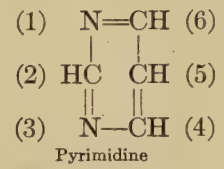




Theobromine occurs in cocoa beans to the extent of 1.5 to 2.4 per cent and in chocolate to the extent of 1 to 2 per cent.

Caffeine occurs in tea, coffee, kola nut, cocoa bean, etc. The coffee bean contains about 1 per cent, tea leaves 1.5 to 2.5 per cent. It is a diuretic and cerebral stimulant and has a pronounced stimulating action on the heart.

The pyrimidine compounds found in nucleic acids are derivatives of the pyrimidine nucleus:

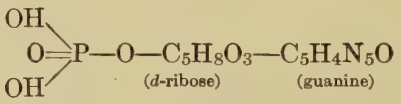


Pyrimidine

The only important derivatives from our standpoint are:

cytosine, 2-oxy-6-amino-pyrimidine;
thymine, 2.6-dioxy-5-methyl-pyrimidine;
uracil, 2.6-dioxy-pyrimidine;
5 *methyl cytosine*, 2-oxy-5-methyl-6-amino-pyrimidine.

The structure of nucleic acids has been largely arrived at by a study of the *mononucleotides*, *i.e.*, compounds containing a single molecule each of phosphoric acid, a carbohydrate, a purine, or a pyrimidine base. Typical mononucleotides are guanylic acid and inosinic acid. These decompose respectively into phosphoric acid, guanine and *d*-ribose, and phosphoric acid, hypoxanthine, and *d*-ribose. The structure of guanylic acid is probably as follows:

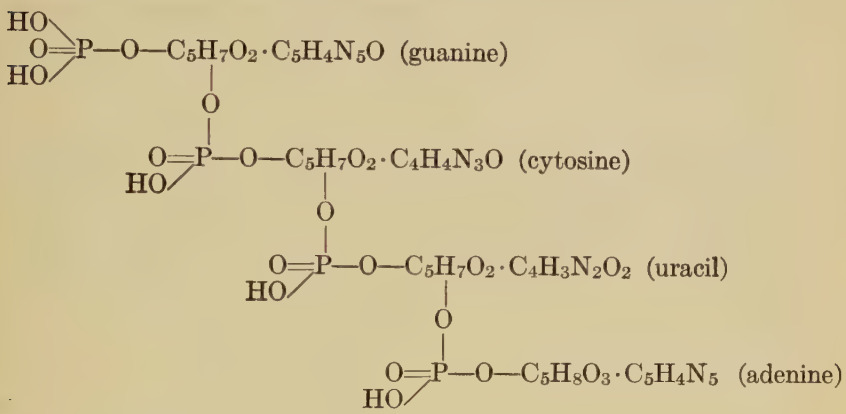


where the union between the carbohydrate and the heterocyclic ring is directly from carbon to nitrogen.

The nucleotides may be converted by the proper reagents into *nucleosides* with the loss of phosphoric acid, a nucleoside being defined as the compound in which a purine or a pyrimidine is joined to a carbohydrate. Various nucleosides have been isolated. Thus, guanosine is a compound composed of one molecule each of guanine and *d*-ribose. Adenosine is composed of a molecule each of adenine and *d*-ribose, etc.

Nucleotides then may be considered as compounds of nucleosides with phosphoric acid, and apparently four nucleotides unite to make a molecule of nucleic acid. Levene and Jacobs, in hydrolyzing yeast nucleic acid, obtained phosphoric acid and four nucleosides containing

guanine, adenine, cytosine, and uracil, respectively. These four nucleotides were apparently condensed with the loss of three molecules of water to form yeast nucleic acid. It is generally considered that the union between the nucleotides is between the carbohydrate groups and phosphoric acid, although certain workers have considered that the union may in some instances be entirely through the phosphoric acid groups. Levene's formula for a hypothetical nucleic acid is as follows:



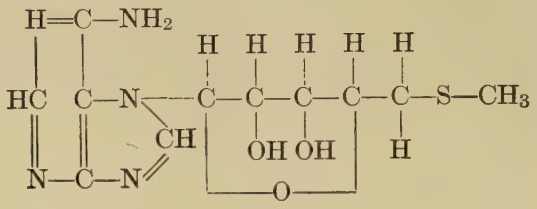
There are still many problems to be solved before we know the exact chemical configuration of the nucleic acids. The possibilities of stereoisomerism are very great. Thus, we know almost nothing in regard to the position of the nitrogen atom which is bound to the α-carbon of the carbohydrate chain. In the case of a purine, there are four nitrogens, any one of which might be involved. In the case of the amino derivatives, the possibilities of union are further increased. Similarly, we do not know which hydroxyl groups in the sugar molecule lose water and form the linkages between the nucleotides. Here again, we have a number of stereoisomers possible. In addition, there is the possibility that other sugars than *d*-ribose may be present. We have already noted that certain nucleic acids yield levulinic acid, indicating the presence of hexoses.

Mandel and Dunham[42] isolated an adenine nucleoside from yeast which apparently had the formula $C_{11}H_{15}N_5O_5$, but which could not be identified with any known nucleoside. Levene[43] later isolated the same compound and reported that the sugar differed from all known keto-

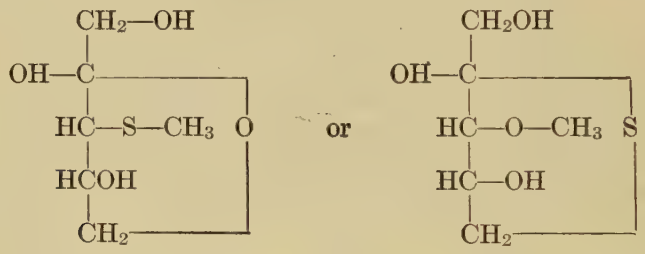
[42] Mandel, J. A., and Dunham, E. K., Preliminary Note on a Purine-Hexose Compound, *J. Biol. Chem.*, 11 : 85–86 (1912).

[43] Levene, P. A., Adenosin Hexoside from Yeast, *J. Biol. Chem.*, 59 : 465–472 (1924).

hexoses. Later, Suzuki, Odake, and Mori[44] isolated an adenine-carbohydrate compound from yeast *and found that the sugar contained sulfur.* They believed it to be a methyl-thio-pentose and ascribed the following formula:



Levene and Sobotka[45] then reinvestigated the nucleoside which Levene had previously isolated, and found that the sugar was not a keto-hexose but that it contained sulfur and was identical with the product isolated by Suzuki, Odake, and Mori. Levene points out, however, that the sugar cannot have the formula suggested above, but that it is probably either



i.e., it is a methylated thio-pentose. Levene and Sobotka note that the $—OCH_3$ or the $—SCH_3$ may be on the fourth instead of the third carbon. This compound is the only sulfur-containing carbohydrate which has been reported in the literature. It is not surprising that Levene considered this to be a keto-hexose, inasmuch as a keto-hexose would have approximately the same molecular weight as the thio-pentose, and accordingly yield approximately the same analysis for carbon and hydrogen. Whether or not this sugar occurs in nucleic acids is still uncertain. Future workers should, however, make tests for the presence of sulfur when they are studying nucleic acids or decomposition products of nucleic acids.

[44] Suzuki, U., Odake, S., and Mori, T., Über einen neuen schwefelhaltigen Bestandteil der Hefe, *Biochem. Z.*, 154 : 278–289 (1924).

[45] Levene, P. A., and Sobotka, H., The Thio-Sugar from Yeast, *J. Biol. Chem.*, 65 : 551–554 (1925).

Rose[46] has recently collected the literature in regard to purine metabolism in man and in animals. He presents in his paper an excellent review of the changes which take place in the various purine and pyrimidine derivatives under the influence of various reagents and enzymes, and also discusses the fate of the various purine derivatives in man and in animals.

The question as to whether or not animals are able to synthesize the purine derivatives needed for nucleic acid formation has not been satisfactorily answered. T. B. Johnson states that since purines can be readily synthesized from pyrimidines, it seems extremely probable that pyrimidines are the precursors of the purines in plants. However, cytosine and uracil have never been found in the free condition in plants but are always combined as nucleosides. On the other hand, purines occur free, probably as an end product of nitrogen metabolism, for example, theobromine and caffeine in plants, and uric acid in man.

As already noted, *chromatin* appears to be almost exclusively composed of protamine nucleate, a protamine combined with nucleic acid. Chromatin is the chemical substance from which *chromosomes* are derived. The nucleic acids form approximately 40 per cent of the solid components of the chromosomes, and as Leathes notes, if we consider that into these chromosomes " are packed from the beginning all that preordains, if not our fate and fortunes, at least our bodily characteristics down to the color of our eyelashes, it becomes a question whether the virtues of nucleic acids may not rival those of amino acid chains in their vital importance." Leathes notes that on the basis of 40 per cent of the nucleic acids in the chromatin, there are approximately half a million molecules of nucleic acid in a sperm cell.

XI. The Lecithoproteins.—The literature is somewhat confused in regard to this class of proteins, some workers feeling that the lecithoproteins are definite compounds, others that they represent mixtures of proteins with lecithin or other phospholipides in varying proportions. Within recent years, however, there appears to be a rather general trend toward viewing the combinations between protein and lipides which occur in certain cells and tissues as representing a conjugated protein in spite of the fact that the lipide fraction can be very largely removed by extraction with fat solvents. Such a viewpoint would probably lead to including the vitellin of egg yolk in the group of lecithoproteins, since it occurs together with large amounts of lecithin in the egg yolk and cannot be completely freed from lecithin except by rather drastic extraction.

[46] Rose, W. C., Purine Metabolism, *Physiol. Rev.*, 3 : 544–602 (1923).

Perhaps the best example of a lecithoprotein which has been studied extensively is the *tissue fibrinogen* which has been investigated by Mills and his coworkers.[47-54] Mills finds that the tissues contain a substance which accelerated the clotting of blood and that this substance is composed of a protein and a phospholipide in the ratio of approximately 58.4 per cent protein and 41.6 per cent of a phospholipide. A large part of the phospholipide can be removed by extraction with fat solvents. A part, however, is very firmly held and is not completely removed except by methods which tend to partially hydrolyze the protein. Mills finds that the purified material possesses extraordinary power in causing blood clotting, and that the lung tissue is very high in the so-called tissue fibrinogen. If the phospholipide is removed from the lipo-protein by fat solvents, neither the protein fraction nor the phospholipide fraction is capable of accelerating blood clotting. If, however, the two extracts are recombined, a large part of the original activity is regained. Tissue fibrinogen is such a powerful blood coagulant as to cause the cessation of hemorrhage even in cases of hemophilia, and may be prepared for either subcutaneous injection or for taking by mouth. If injected intravenously, death ensues, due to the formation of clots in the blood vessels. Mills believes that the presence of such materials in the tissues is the protective agency which the body provides against excessive hemorrhage.

Mills and Mathews have studied the mechanism of the clotting of blood and find that both in the normal clotting process and the clotting process induced by tissue fibrinogen, there is the interaction of both protein and phospholipide. The following chart prepared by Mills and Mathews indicates the two mechanisms of blood clotting.

[47] Mills, C. A., The Activity of Lung Extract, as Compared to Extracts of Other Tissues, in Inducing Coagulation of the Blood, *J. Biol. Chem.*, 40 : 425–433 (1919).

[48] Mills, C. A., Chemical Nature of Tissue Coagulins, *J. Biol. Chem.*, 46 : 135–165 (1921).

[49] Mills, C. A., The Action of Tissue Extracts in the Coagulation of Blood, *J. Biol. Chem.*, 46 : 167–192 (1921).

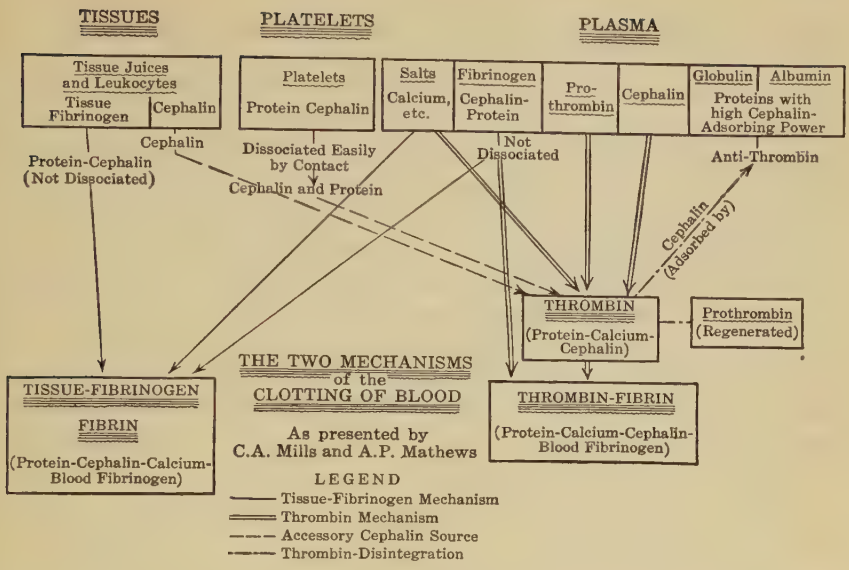
[50] Mills, C. A., Raap, G., and Jackson, D. E., A Note on the Relation between the Blood-Coagulating and the Smooth Muscle-Contracting Properties of Tissue Extracts, *J. Lab. Clin. Med.*, 6 : 374–388 (1921).

[51] Mills, C. A., and Guest, G. M., The Role of Tissue Fibrinogen (Thrombokinase) in Fibrin Formation and Normal Clotting, *Am. J. Physiol.*, 57 : 395–419 (1921).

[52] Mills, C. A., Blood Clotting Studies in Hemophilia, *Am. J. Physiol.*, 76 : 632–641 (1926).

[53] Mills, C. A., The Manner of Action of the Protein Fraction of Tissue Fibrinogen as a Blood Anticoagulant, *Am. J. Physiol.*, 76 : 651–659 (1926).

[54] Mills, C. A., Considerations of the Problem of Blood Clotting, *Am. J. Med. Sci.*, 172 : 501–510 (1926).



Troensegaard and Koudahl[55] have recently reported the presence of cholesterol as a group associated with the globulin of blood serum.

It seems probable that in many instances the proteins existing in the tissues may be associated with either fats, lipides, or with fatty acids. However, in our method of purification of the protein we remove these groups by the use of fat solvents. This is perhaps simply a restatement of the remarks already credited to Abderhalden where he notes that the proteins prepared and purified for chemical study may in many instances bear little resemblance in their physical properties to the proteins as they actually exist in the cells and tissues.

XII. Derived Proteins.—Derived proteins, as already noted, are proteins which have been chemically altered by manipulation subsequent to their isolation. They include such groups as the coagulated proteins, the halogenated proteins, the nitrated proteins, the formaldehyde proteins, deaminized proteins, racemized proteins, and proteans, etc. The literature in this field is so voluminous that mention can be made of only a few of the reactions involved.

Coagulated Proteins.—Perhaps the best known of the coagulated proteins is the heat-coagulated egg white. When a concentrated solution of egg white is heated, the proteins coagulate and the entire mass sets to a more or less rigid gel. If, however, the egg white has previously been

[55] Troensegaard, N., and Koudahl, B., Cholesterin als prostethische Gruppe im Serum-Globulin, *Z. physiol. Chem.*, 153 : 111–118; 157: 62–63 (1926).

diluted, gel formation does not occur, the protein separating instead in the form of flocs.

We have already noted that coagulation occurs at the interface between an egg albumin solution and air. In this case, surface energy brings about the same sort of coagulation that heat energy does in the heat-coagulation of the albumin. Various other forms of energy likewise will induce coagulation. Thus, for example, coagulation may be brought about by irradiating with ultraviolet light,[56-59] with the rays of a radium salt,[60] or by high pressures,[61] as well as by the application of heat.[62] In each instance energy is added to the system.

There is rather general disagreement among the various workers as to what actually occurs when a protein is coagulated. Some workers believe that the initial reaction is one of dehydration in which free amino and carboxyl groups within the protein molecule react, with the formation of an anhydride structure,

$$HOOC—(R)_x—NH_2 \rightarrow \underset{\_\_\_\_\_}{CO—(R)_x—NH} + H_2O$$

where $(R)_x$ represents the mass of the protein molecule. This viewpoint is diametrically opposed to the viewpoint of others who insist that the initial reaction appears to be an incipient hydrolysis, this argument being based upon the fact that there is always a small amount of non-heat-coagulable nitrogen remaining in the solution from which the heat-coagulated protein has been removed by filtration. This fact is taken as evidence that some simpler decomposition products have been split off from the original protein molecule.

One group of workers regards protein coagulation as an initial dehydration of a hydrated micelle, while another group believes that the colloidal micelle has taken on additional water molecules and increased the hydration. Still another group has suggested that coagulation may

[56] Clark, J. H., The Action of Ultraviolet Light on Egg Albumin in Relation to the Isoelectric Point, *Am. J. Physiol.*, 61 : 72–79 (1922).

[57] Clark, J. H., Studies on Radiated Proteins. I. Coagulation of Egg Albumin by Ultraviolet Light and Heat, *Am. J. Physiol.*, 73 : 649–660 (1925).

[58] Bovie, W. T., A Preliminary Note on the Coagulation of Proteins by Ultraviolet Light, *Science*, 37 : 24–25 (1913).

[59] Bovie, W. T., The Temperature Coefficient of the Coagulation Caused by Ultraviolet Light, *Science*, 37 : 373–375 (1913).

[60] Fernau, A., and Pauli, W., Über die Einwirkung der durchdringenden Radiumstrahlung auf anorganische und Biokolloide, I., *Biochem. Z.*, 70 : 426–441 (1915).

[61] Bridgman, P. W., The Coagulation of Albumen by Pressure, *J. Biol. Chem.*, 19 : 511–512 (1914).

[62] Note particularly, Berczeller, L., Stalgmometrische Studien an kolloiden und krystalloiden Lösungen, *Biochem. Z.*, 53 : 215–231 (1913).

well be synonymous with polymerization, several protein molecules combining into a larger polymer or aggregate.

The only point at which there appears to be unanimity of opinion is that *the coagulation process occurs in two stages*, a preliminary stage of *sensitization* followed by a secondary stage in which *flocculation* takes place. This appears to be true irrespective of the means by which coagulation is brought about. Thus, it is possible to prepare an albumin sol which is salt-free, which can be irradiated with ultraviolet light, or heated above the heat-coagulating temperature without the flocking of the protein. Protein sols which have been so treated are in a highly sensitive condition and flocs are immediately produced when traces of electrolytes are added to such systems. There would seem to be a striking similarity between such systems and a lyophilic → lyophobic sol transformation. The lyophilic sols are not sensitive to the action of traces of electrolytes, whereas lyophobic sols are exceedingly sensitive. Unaltered protein sols are not sensitive to the action of electrolytes, whereas protein sols which have been sensitized by either heat or irradiation become very sensitive to the action of electrolytes.

Coagulation can be produced by exposure to ultraviolet light even at 0°. Bovie points out that the coagulation by ultraviolet light has a negligible temperature coefficient, the rate of coagulation being nearly as great at 0° as at higher temperatures. On the other hand, if an albumin solution is rayed for a time only partially long enough to induce coagulation, it is found that the temperature at which it will heat-coagulate has been markedly decreased. It is thus possible to ray an egg albumin solution at 0° for a few minutes and have it remain in an apparently unchanged condition as long as it is kept at 0°. If, however, the temperature is allowed to rise to room temperature, it spontaneously flocculates, or if sufficiently concentrated, sets to a gel.

Miss Clark notes that the change by ultraviolet light is more or less analogous to an albumin → globulin transformation. Inasmuch as the protein in the sols sensitized by ultraviolet radiation can be precipitated by half saturation with ammonium sulfate, thus behaving as globulins. The altered protein, however, is not peptized by dilute salt solutions as it should be if a true albumin → globulin transformation had taken place.

Although a number of authors have claimed that if coagulation had proceeded only to the point of incipient turbidity, the reaction could be reversed by cooling the solution or removing it from the source of radiation and allowing it to stand for a time, Adolf,[63] insofar as the author is

[63] Adolf, Mona Spiegel, Hitzeveränderungen des Albumins, *Biochem. Z.*, 170 : 126–172 (1926).

aware, is the only person to claim a successful reversal of the complete heat coagulation process. She studied serum albumin purified by electrodialysis and found that the hydrogen ion concentration of the sol decreased from 5.3×10^{-6} to $2.84\text{–}2.71 \times 10^{-7}$, and the specific electrical conductivity increased from 5.46×10^{-6} to 7.33×10^{-6}, on heat coagulation. By dissolving the coagulated protein in dilute sodium hydroxide and subsequently removing the alkali by electrodialysis, she states that she regenerated a sol having all the properties of the initial serum albumin sol, including hydrogen ion concentration, specific electrical conductivity, optical rotation, temperature of coagulation, and protective value toward gold and mastic sols. If this be the case, it would appear that heat denaturization is not as deep-seated a process as other workers have indicated.

The whole problem of protein coagulation requires further investigation but it seems probable that the change which is involved is so subtle as not to be readily amenable to the relatively crude methods used in protein research.

Halogenated Proteins.—When the halogens, chlorine, bromine, or iodine, are introduced into the protein, the question always arises as to whether or not the method of halogenation involves a partial hydrolysis of the protein. In most instances, this is undoubtedly the case. In general, substitution takes place on the aromatic nuclei, notably the benzene rings of tryptophane, tyrosine, and to some extent that of phenylalanine. The same may be said for the nitrated proteins. The Millon and the Adamkiewicz reactions, as a rule, are negative, the characteristic hydrolytic products being the halogenated or nitrated aromatic radicals, particularly that of tyrosine, although the nitrated proteins yield nitroarginine.

Formaldehyde Proteins.—Proteins unite with formaldehyde to form compounds having very different properties from the original protein. Thus, egg albumin treated with formaldehyde is no longer coagulable by heat. Most of the work on the formaldehyde proteins has been carried out using casein, and the manufacture of artificial ivory, galalith, or casein plastics, involving the union of formaldehyde and casein, has resulted in the building up of a large chemical industry, the more or less chemically reactive casein being converted into a relatively inert horn-like mass. A discussion of this process has been given by Brother.[64] Little is known in regard to the chemical reactions which are involved. Yeast and yeast residues, or the protein of the soybean have been used to replace casein, forming similar " artificial ivories."

[64] Brother, G. H., Casein Plastics, Chapter VI, in Sutermeister's Casein and Its Industrial Applications, Chemical Catalog Company, Inc., New York (1927).

Deaminized Proteins.—Deaminized proteins, prepared by the action of nitrous acid on the protein, have been rather extensively studied. The resulting products contain no free $—NH_2$ groups and show quite altered chemical properties toward acids. Insofar as their hydrolytic products are concerned, the only notable change is that lysine can no longer be isolated, the reaction between the protein and the nitrous acid resulting in the elimination of the ϵ-amino group.

The Proteans.—The change of certain globulins to an insoluble modification, known as a protean, has already been noted. Edestin of hempseed, excelsin of the Brazil nut, and myosin of the muscle fibers are readily transformed from a protein which is easily peptized by 10 per cent sodium chloride to proteans which are no longer peptized. Here again, we do not know the nature of the chemical change which is involved. Osborne[65] believes that this transformation involves hydrolysis and that the edestan may be regarded as the first stage in the hydrolysis of edestin. He finds that the transformation is accelerated by the presence of acids and that the various acids produce in a given time very different amounts of the edestan. The figures which he gives indicate that the hydrogen ion is the active catalyst, although his work was done prior to the general use of hydrogen ion control. Table LI

TABLE LI

Showing Effects of Reagents on the Edestin → Edestan Transformation
(Data of Osborne)

Solution	Temperature, ° C.	Time, Hours	Edestin Transformed to Edestan, Per Cent
10% NaCl	20	6	2.16
$H_2O + CO_2$	20	6	6.75
H_2O	20	6	4.32
H_2O	30	6	7.11
H_2O	50	6	29.00
N/100 HCl	20	24	79.02
N/143 HCl	20	3	29.80
N/143 HCl	20	20	33.55
N/143 HCl	20	*	19.29
†N/143 H_3PO_4	20	*	16.02
N/143 $H \cdot C_2H_3O_2$	20	*	5.65

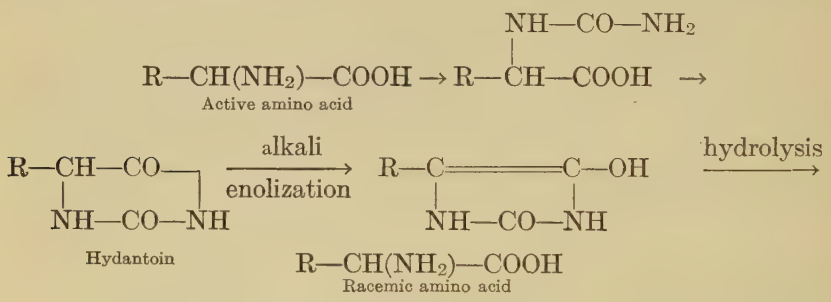
* Time identical for all three acids. Exact time not stated.
† Calculated as monovalent.

[65] Osborne, T. B., Ein hydrolytisches Derivat des Globulins Edestin und sein Verhältniss zu Weyl's Albuminat und zur Histongruppe, *Z. physiol. Chem.*, 33 : 225–239 (1901).

lists some of the results which he obtained. Osborne notes that the chemical analysis of edestan is within experimental error of the chemical analysis of the edestin from which it was derived.

The author is not aware of any recent extensive study dealing with the protein → protean transformation.

Racemized Proteins.—Dakin[66] observed that when proteins were dissolved in dilute alkali, there was a progressive fall in the specific optical rotation with time, to a much lower value which eventually became constant. He observed a similar change in the optical rotation of hydantoins prepared from optically active amino acids and noted that when hydantoins which had stood for a time in an alkaline solution were hydrolyzed, they yielded racemic amino acids rather than the active amino acids. He explains the change produced by the alkali by a keto ⇌ enol isomerism, the hydrogen migrating from the α-carbon atom to the carbonyl group, forming a double bond, as shown in the accompanying structural formulae. Later when hydrolysis occurs and the amino acids are regenerated, the double bond no longer exists, but since either one of the unions of the double bonds may later become attached to hydrogen, we would expect to find not the active but equal amounts of *d* and *l* acids to be formed.



In working with gelatin, Dakin found that a similar racemization occurred and when the gelatin was later hydrolyzed, he found that certain of the amino acids were optically inactive, whereas others were optically active. The inactive amino acids were leucine, aspartic acid, arginine, histidine, and phenylalanine. Part of the alanine was active; part was inactive. All of the proline, glutamic acid, and lysine which he isolated was optically active. He believes that the reason that certain amino acids were racemized, whereas others were not, depends upon the position of the amino acid in the protein molecule, and notes that only those amino acids, where both the $—NH_2$ group and the —COOH are

[66] Dakin, H. D., The Racemization of Proteins and Their Derivatives Resulting from Tautomeric Change. Pt. I, *J. Biol. Chem.*, 13 : 357–362 (1912).

combined in the protein molecule, undergo racemization. If either of these groups are free, racemization does not occur and accordingly the optically active acid can be isolated from the racemized protein. According to this viewpoint, the inactive amino acids are situated somewhere in the interior of the chain making up the protein molecule, whereas those which are not racemized are on the ends of the peptide chain.

Later, Dakin and Dale[67] compared the albumins of hens' eggs and ducks' eggs in regard to racemization and to acids which they yielded on hydrolysis. Only three amino acids were found to show characteristic differences. Leucine from racemized albumin from hens' eggs was almost completely racemic, whereas leucine isolated from the racemized duck albumin was almost completely active. Aspartic acid from the duck albumin was completely inactive, whereas that from hen albumin was partially active. Histidine from hen albumin was completely inactive, from duck albumin almost completely active. They believe that these observations indicate structural differences in the two albumins.

Woodman[68] investigated the corresponding proteins in cow and ox serum and in cow's milk and colostrum, by the method of protein racemization. He concludes that the globulins of cow and ox serum and of colostrum are identical, that the albumin of milk is identical with the albumin of colostrum, but that lactalbumin and serum albumin are not identical.

Dakin and Dudley[69] studied racemized casein, and Dudley and Woodman[70] repeated the work of Dakin and Dudley, comparing casein from cow's milk and casein from the milk of sheep. In the case of casein from cow's milk, all of the amino acids isolated were racemized, with the exception of proline and part of the valine and leucine. In the case of casein from sheep's milk, lysine, proline, and tyrosine, were all optically active, glutamic acid, leucine, and valine partly active. They, there-

[67] Dakin, H. D., and Dale, H. H., Chemical Structure and Antigenic Specificity. A Comparison of the Crystalline Egg-Albumins of the Hen and the Duck, *Biochem. J.*, 13 : 248–257 (1919).

[68] Woodman, H. E., A Comparative Investigation of the Corresponding Proteins of Cow and Ox Serum, Cow's Colostrum and Cow's Milk by the Method of Protein Racemisation, *Bioch. J.*, 15 : 187–201 (1921).

[69] Dakin, H. D., and Dudley, H. W., The Racemization of Proteins and Their Derivatives Resulting from Tautomeric Change. Pt. II. The Racemization of Casein, *J. Biol. Chem.*, 15 : 263–269 (1913).

[70] Dudley, H. W., and Woodman, H. E., The Specificity of Caseinogens. A Comparative Study of the Caseinogens of the Cow and the Sheep, *Biochem. J.*, 9 : 97–102 (1915).

fore, reached the conclusion that cow and sheep caseins contained the amino acids linked in different positions.

Dakin and Dudley[71] attempted to isolate tryptophane from racemized casein by tryptic digestion and found that neither trypsin, erepsin, nor pepsin would attack the racemized protein; neither was it digested *in vivo*, and when it was fed to dogs, the racemized protein was eliminated unchanged in the feces. They likewise note that the racemic casein was not attacked by putrefactive bacteria during a ten-day interval, but following that time was very slowly attacked. They point out that all artificial polypeptides which have been synthesized have been subjected at some stage or other to alkaline conditions, and suggest that possibly the failure of enzymes to attack certain of these synthetic products may be due to some change similar to the change which has taken place in protein racemization. They also note that in the separation of proteins from biological materials, precautions must be taken not to subject them to the presence of alkalies for any extended time.

Ten Broeck[72] prepared racemic egg albumin by allowing egg albumin to stand at 37° for three weeks in contact with 0.5 N sodium hydroxide. He states that the product which was isolated differed chemically from egg albumin only in rotatory power. On an attempt to immunize animals with this racemic protein, he found that it had no immunological power. It neither sensitized nor intoxicated; no antibodies were formed as shown by various tests. These observations of Ten Broeck support Vaughan's idea (*vide infra*) that a splitting of the protein must take place before immunological reactions occur. Obviously, if racemic proteins are not attacked by enzymes, no splitting can take place.

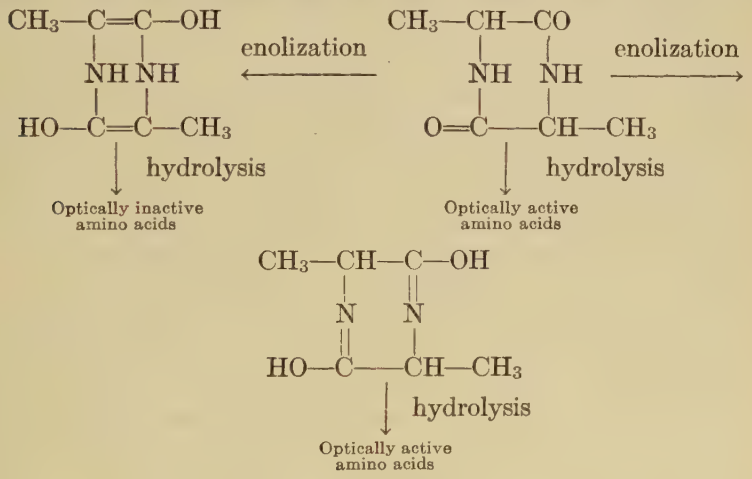
The above discussion and the papers cited would lead one to believe that a racemic protein is merely a protein in which certain shifts in linkages have taken place with a concomitant change in optical rotation, and that racemic proteins are somewhat analogous to coagulated proteins and proteans. However, this appears to be the wrong viewpoint. In most instances, the racemic product which is isolated amounts to only a fraction of the weight of protein which is originally taken. Thus, 20 to 30 grams of racemic casein can be isolated if 100 grams of casein have been acted upon by the 0.5 N sodium hydroxide. This fact indicates that a large part of the casein has undergone deep-seated hydrolysis, and one of the hydrolytic products, *i.e.*, the racemized "protein," is still capable of being readily isolated. The question arises, therefore, as to

[71] Dakin, H. D., and Dudley, H. W., The Action of Enzymes on Racemized Proteins, and Their Fate in the Animal Body, *J. Biol. Chem.*, 15 : 271–276 (1913).

[72] Ten Broeck, C., The Non-Antigenic Properties of Racemized Egg Albumin, *J. Biol. Chem.*, 17 : 369–375 (1914).

whether the chemical analysis of this protein fraction can be compared with the chemical analysis of the original protein, in drawing valid conclusions.

Levene and Pfaltz[73, 74] have investigated the action of alkali on diketopiperazines and polypeptides. They find that the dipeptide, *d*-alanyl *d*-alanine, is not racemized by alkali. The anhydride, however, changes from an optical rotation of $+17.5°$ to $-16.0°$, at which point 50 per cent of the nitrogen is amino nitrogen, *i.e.*, hydrolysis of the polypeptide has taken place. They note that the diketopiperazine ring must remain in contact with the alkali for an appreciable period of time before it hydrolyzes, in order to form intermediate products which on hydrolysis yield optically inactive acids. It will be noted that there are two enol forms of the dimethyl-diketopiperazines, as shown in the following diagram, only one of which will yield the optically inactive amino acids.



Levene and Pfaltz note that even when the tripeptide, glycyl-*l*-alanyl-glycine and the tetrapeptide, glycyl-glycyl-*l*-alanyl-glycine, were allowed to stand in contact with alkali, no racemization occurred during the first forty-eight hours, but on long standing, there was a slight decrease in optical rotation, the maximum racemization never exceeding 10 per cent and even in these cases hydrolysis reached 80 per cent or more. They note that there is no way of determining whether racemization precedes

[73] Levene, P. A., and Pfaltz, M. H., Studies on Racemization. Action of Alkali on Dextro-Alanyl-Dextro-Alanine Anhydride, *J. Biol. Chem.*, 63 : 661–668 (1925).

[74] Levene, P. A., and Pfaltz, M. H., Studies on Racemization, III. Action of Alkali on Glycyl-Levoalanyl-Glycine and on Glycyl-Glycyl-Levoalanyl-Glycine, *J. Biol. Chem.*, 68 : 277–283 (1926).

or follows the hydrolysis, and point out that perhaps the racemization of proteins may be the racemization of diketopiperazine compounds rather than the long peptide chains which Dakin postulates. Further work will have to be done before either possibility is proven.

Acid and Alkali Albumins.—These are perhaps best typified by Paal's[75] protalbinic and lysalbinic acids which have already been noted as examples of protective colloids. We should perhaps add to this group the products prepared by Vaughan's method[76] of partially hydrolyzing proteins in alcoholic solution with sodium hydroxide. In this way, proteins can be separated into two fractions, an alcohol-soluble portion and an alcohol-insoluble residue. The alcohol-soluble portion was found by Vaughan to be exceedingly poisonous when injected intravenously, exceeding in its toxic action even such drugs as strychnine. No adequate explanation for the high degree of toxicity has been offered. Vaughan notes, however, that gelatin appears to be the only protein which does not yield the toxic fraction. We have already noted that gelatin is deficient in a number of amino acids, those containing aromatic nuclei, and that it does not induce the usual immunological reactions. Vaughan believes the immunological reactions are, at least in part, induced by a hydrolytic cleavage in the animal body, more or less similar to the hydrolytic cleavage which takes place in an alcoholic sodium hydroxide solution, and that the toxins liberated in the anaphylactic reaction are similar, if not identical, to his protein poisons.

White and Avery[77] have shown that similar protein poisons can be prepared from the proteins of the tubercle bacillus by Vaughan's method.

Relatively little work from the chemical standpoint has been done with these products. Miss Wheeler[78] has shown that they are complex, and has isolated a number of amino acids from the various preparations.

[75] Paal, C., Ueber die Einwirkung, ätzender Alkalien auf Eialbumin, *Ber.*, 35 : 2195–2206 (1902).

[76] Vaughan, V. C., and Novy, F. G., Cellular Toxins, Lee Brothers, (1902).

[77] White, B., and Avery O. T., The Action of Certain Products Obtained from the Tubercle Bacillus. A. Cleavage Products of Tuberculo-Protein Obtained by the Method of Vaughan. I. The Poisonous Substance, *J. Med. Res.*, 26 : 317–356 (1912).

[78] Wheeler, S. M., A Study of the Chemistry of Bacterial Cellular Proteins, *J. Biol. Chem.*, 6 : 509–552 (1909).

CHAPTER XVII

THE REACTIONS OF PROTEINS WITH ACIDS AND BASES

From the preceding discussions of the chemical nature of the proteins, it can be readily seen that the protein molecule possesses both acidic and basic groups and accordingly must be looked upon as a complex amphoteric substance. We have likewise noted that changes in hydrogen ion concentration in many instances bring about molecular rearrangements, such as a shift in the keto $\rightleftharpoons$ enol isomerism, whereby additional reactive groups are formed within the protein molecule. We have similarly noted that proteins in solution show the characteristic properties of lyophilic sols and that the colloidal micelles may be either positively or negatively charged, depending upon the hydrogen ion concentration of the dispersions medium.

It is not surprising, therefore, to find that proteins undergo reactions with acids and bases, and with certain salts, notably the salts of the heavy metals. These reactions have been studied by a great many workers and have given rise to a very voluminous literature. In order to discuss this literature adequately, it would require far more space than can be justified in the present connection. Casual mention can be made of only a few of the more important considerations.

In spite of the large amount of work which has been carried out in this field, the various workers in this field are far from agreement. The workers may be classified by and large into three groups.

One group of workers insists that the reactions that take place between proteins and acids, bases, or salts are purely chemical reactions determined by primary valence forces of the free amino groups, the free carboxyl groups, or similar active groups within the protein molecule, and that the reactions are stoichiometrical in character, with colloidal reactions playing no appreciable role. This school of thought received a great impetus from the work of Loeb and is being ably continued since his death, through the activities of E. J. Cohn, D. I. Hitchcock, John Arthur Wilson, and C. L. A. Schmidt, to mention only a few of the outstanding workers.

Another group of workers views the reactions of proteins as the reactions of a lyophilic colloid system and support the view that the

hydrogen ions, the hydroxyl ions, and the ion interchange with salt solutions is probably largely due to the forces of adsorption characteristic of lyophilic colloid interfaces. To this group the colloidal properties of the system far outweigh the chemical forces of primary valence.

A third group of workers may well include those who, while admitting that the forces of primary valence play a role, nevertheless insist that the colloidal properties of a lyophilic system must be taken into account before an adequate knowledge of protein chemistry can be secured. This group insists that at least under certain conditions the colloidal forces outweigh the chemical forces of primary valence and tend in any event to modify the forces of primary valence.

Cohn[1] has presented an able review of the physical chemistry of the proteins, emphasizing in particular the viewpoint that the reaction between proteins and acids and bases are stoichiometrical reactions determined by the forces of primary valence.

We have already noted in the consideration of the Donnan equilibrium that the establishment of such an equilibrium is independent of the nature of the process which causes the formation of a non-diffusible ion. Similarly, it is immaterial in many instances whether a charged particle is formed by the process of ionization or by the colloidal process of adsorption. In either instance the charged micelle will behave as an ion, and as such will obey the physico-chemical laws characteristic of an ionized system. It is this fact that makes it difficult to decide between the colloidal viewpoint and the purely stoichiometrical viewpoint in regard to protein compounds.

The author has supported the view that protein systems must from the very nature of the system show the typical reactions that are characteristic of lyophilic colloid systems, admitting at the same time that if a protein is characterized by possessing free $-NH_2$ and free $-COOH$ groups, such groups may be expected to react chemically with acids and bases, respectively. The earlier literature in regard to acid and alkali binding has been reviewed by Hoffman and Gortner[2] and extended to a comparative study of the prolamines, in which study casein and fibrin were used as reference proteins. The earlier workers on acid and alkali binding used various types of physico-chemical technic. These may be divided as follows:

The first method is the direct method of *precipitating out a protein*

[1] Cohn, E. J., The Physical Chemistry of the Proteins, *Physiol. Rev.*, 5 : 349–437 (1925).

[2] Hoffman, W. F., and Gortner, R. A., Physico-Chemical Studies of Proteins. I. The Prolamines—Their Chemical Composition in Relation to Acid and Alkali Binding, Colloid Symposium Monograph, Vol. 2, pp. 209–368 (1925).

compound. This has been used by a number of workers, particularly those who have been dealing with the calcium compounds of casein, casein being titrated with calcium hydroxide to some definite end point as determined by some particular indicator, and then alcohol added to the system until the casein, containing a certain amount of calcium, precipitates. Using this method, one can obtain casein precipitates with different calcium contents, depending upon the indicator used. When litmus is the indicator, only approximately one-half as much calcium is precipitated with the casein as when phenolphthalein is used as the indicator. Accordingly it has been suggested that casein combines with different quantities of calcium to form the so-called " monocalcium caseinate," and the so-called " dicalcium caseinate."

The author believes that this method is incapable of yielding definite results. The indicator chosen will determine to a very considerable extent the amount of calcium hydroxide which must be added to a given amount of casein before the neutral point is reached. Accordingly it would be possible to precipitate an entire range of casein-calcium " compounds," depending upon the indicators which were chosen, the range being from pure casein containing no calcium to casein saturated with calcium and contaminated with precipitated calcium hydroxide. In addition, the " compound " precipitated with alcohol may or may not have a ratio of casein to calcium, identical with the " compound "present in the solution prior to the addition of the alcohol.

A second method of indicating the presence of protein compounds was by *dissolving water-insoluble substances in protein solutions.* Thus, casein decomposes calcium carbonate when ground with precipitated calcium carbonate in water solution. Edestin or casein dissolve a certain amount of water-insoluble alkaloids, such as strychnine, or certain proteins dissolve freshly precipitated copper hydroxide to form sols containing the protein and calcium, strychnine, or copper, respectively. Edestin will dissolve approximately 35 per cent of its weight of copper from freshly precipitated copper hydroxide. The question arises here as to whether these reactions are the reactions characteristic of the hydrogen ions formed by the dissociation of —COOH groups or whether they are, in part at least, the reactions characteristic of peptization.

It would seem to the author that we do not have a clear-cut effect of the hydrogen ion, particularly in view of such solubility effects as have been noted for salt solutions by Kruyt and Robinson (cf. Table XXIX). The protein sols may act as peptizing agents and undoubtedly will act as protective colloids, thus favoring peptization, so that at least a part of the reactions between proteins and insoluble substances may well be attributed to the colloidal behavior of the protein system. This statement

should not be taken to mean that the author does not recognize that there are free carboxyl groups in the proteins and that these play a role in such reactions. The only point that it is desired to emphasize is that no method as yet available delimits such actions from reactions characteristic of colloid systems.

A third method which has been proposed is to study *precipitates formed by the interaction of two soluble substances*, one containing a protein radical. Thus, protein dispersed in hydrochloric acid, when added to a metallic phosphotungstate, precipitates a protein phosphotungstate complex. The latter is possibly a chemical compound. Equally possibly it is an adsorption complex. Again we have a method where we cannot sharply separate the colloidal and the stoichiometrical reactions.

Electrical conductivity studies were first used by Sjöqvist[3, 4] when he used the technic of adding increasing amounts of protein to a constant amount of acid until a constant electrical conductivity was reached. Figure 110 shows certain of his results, using egg albumin and 0.025 N hydrochloric acid. It will be noted that a constant value is reached at approximately 4.25 grams of protein per 100 cc. of acid.

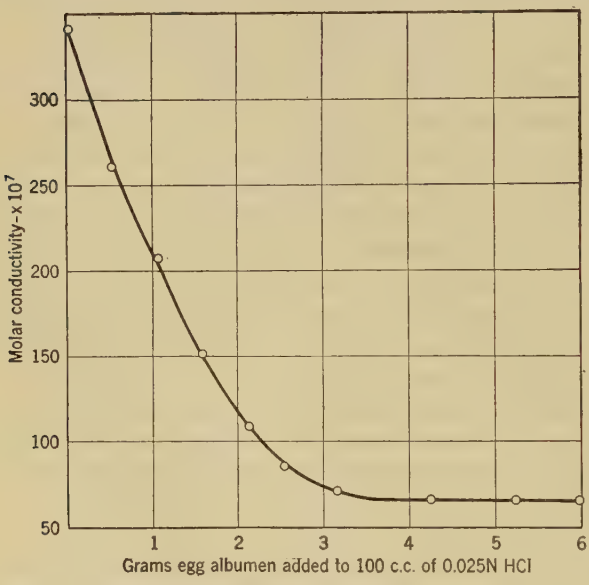


FIG. 110.—Showing the change in the electrical conductivity of a dilute hydrochloric acid solution to which increasing amounts of protein (egg albumin) were added. (Data of Sjöqvist.)

Hoffman and Gortner report similar studies for the prolamines and casein and fibrin. They were unable, however, to correlate their findings

[3] Sjöqvist, J., Physiologisch-chemische Beobachtungen über Salzsäure, *Skand. Arch. Physiol.*, 5 : 277–375 (1895).

[4] Sjöqvist, J., Berichtigungen und Zusätze zu meinem Aufsatz: Physiologisch-chemische Beobachtungen über die Salzsäure, *Skand. Arch. Physiol.*, 6 : 255–261 (1895).

in any definite way with the chemical composition of the proteins which were studied.

Until more is known in regard to the mobility of the specific protein ions and accordingly the size of these ions, as well as the activity of the ions, one cannot interpret conductivity data in terms of definite compound formation. Here again, it may well be that adsorption of either the anion or the cation accounts for at least a part of the apparent binding of the acid.

The cryoscopic method was first employed by Bugarszky and Liebermann[5] who found that the addition of 6.4 grams of egg albumin to 100 cc. of 0.5 N hydrochloric acid reduced the depression of the freezing point of the acid solution to approximately 50 per cent of its initial value.

Barnett[6] made a comparative study of acid and alkali binding, using electrical conductivity, cryoscopic, and potentiometric technic, and reached the conclusion that the cryoscopic technic is the least valuable, due to the small depressions contributed by the protein ions or micelles. In a number of instances the depression obtained was not appreciably greater than the experimental error of the method.

Another method is the *catalytic effect of hydrogen and hydroxyl ions.* Some reaction is selected which is catalyzed by one of these ions, for example, the inversion of sucrose by hydrogen ions (Hoffmann[7,8]) or the saponification of an ester by hydroxl ions (Wintgen and Krüger[9]). When a protein is added to such a system, the rate of catalysis is changed, and it is concluded that hydrogen ions have been removed from that system. Here again, the method gives no indication as to the mechanism whereby hydrogen or hydroxyl ions are removed from the solution.

Other methods have been proposed, such as *the masking of the toxicity of a heavy metal,* such as barium, mercury, copper, or silver, by proteins, a higher concentration of these toxic metals being required when their solutions are mixed with proteins. Here again, we cannot distinguish between a chemical compound and an adsorption complex.

The method which has been most generally employed in recent years

[5] Bugarszky, S., and Liebermann, L., Ueber das Bindungsvermögen eiweissartiger Körper für Salzsäure, Natriumhydroxyd und Kochsalz, *Pflüger's Arch. ges. Physiol.*, 72 : 51–74 (1898).

[6] Barnett, H. M., A Study of Acid and Base Binding by Proteins, Thesis for the M.S. degree, filed in the Library of the University of Minnesota, Minneapolis (1927),

[7] Hoffmann, F. A., Erkennung und Bestimmung der freien Salzsäure im Magensaft, *Z. klin. Med.*, 10 : 793–796 (1889).

[8] Hoffmann, F. A., Weitere Bemerkungen über Salzsäure im Mageninhalt, *Z. klin. Med.*, 11 : 521–524 (1890).

[9] Wintgen, R., and Krüger, K., Ueber das Gleichgewicht Gelatine-Salzsäure, *Koll. Z.*, 28 : 81–89 (1921).

is the *potentiometric method*, involving the measurement of the change in hydrogen or hydroxyl ion concentration in acid or alkali solutions to which proteins have been added. This method was first used by Bugarszky and Liebermann[10] who conclude that chlorine ions, as well as hydrogen ions, are bound by the protein. Blasel and Matula[11] have proposed the formula,

$$n = \mathrm{N} - \frac{\mathrm{C_H}}{\alpha} \tag{115}$$

where n = the amount of acid or alkali bound;
N = the original normality of acid or alkali;
$\mathrm{C_H}$ = the measured hydrogen ion concentration of the protein-acid or protein-alkali solution at equilibrium;
α = the dissociation constant as calculated from specific conductivity data,

for calculating the amount of acid or alkali which is bound by potentiometric methods.

Later, Pauli and Spitzer[12] proposed the formula for strong acids and bases,

$$n = \mathrm{N} - \frac{\mathrm{C_{OH}}}{\alpha} \tag{116}$$

and the formula for weak bases,

$$n = \frac{\mathrm{K(N} - \mathrm{C_{OH}}) - (\mathrm{C_{OH}})^2}{\mathrm{K} - \mathrm{C}_H} \tag{117}$$

where the symbols have the same significance as in formula (115).

Lloyd and Mayes[13] proposed the formula,

$$n' = \mathrm{N} - \frac{(\mathrm{H^+})\mathrm{corr.}}{\alpha} \tag{118}$$

[10] Bugarszky, S., and Liebermann, L., Ueber das Bindungsvermögen eiweissartiger Körper für Salzsäure, Natriumhydroxyd und Kochsalz, *Pflüger's Arch. ges. Physicl.*, 72 : 51-74 (1898).

[11] Blasel, L., and Matula, J., Untersuchungen über physikalische Zustandsänderung der Kolloide. XVI. Versuche am Desaminoglutin, *Biochem. Z.*, 58 : 417–450 (1913).

[12] Pauli, W., and Spitzer, A., quoted by Pauli, "Colloid Chemistry of the Proteins, Pt. I., pp. 93–95, (translated by P. C. L. Thorne), J. and A. Churchill, London, (1922).

[13] Lloyd, D. J., and Mayes, C., The Titration Curve of Gelatine, *Proc. Roy. Soc.*, 93B : 69–85 (1922).

where n' = the normality of acid bound;
N = the original normality of acid;
(H^+) corr. = $\sqrt{(H^+)(Cl^-)}$;
α = the degree of ionization of the acid as determined by conductivity data.

When Hoffman and Gortner undertook their study of the prolamines, they found that α, the degree of ionization of the acid as determined by conductivity data, was not the same as α', the degree of ionization determined potentiometrically. In the preceding formulae (115), (116), (117), and (118), the assumption had been made that the degree of ionization of an acid could be accurately determined by conductivity data. Hoffman and Gortner accordingly calculated their values for acid and alkali binding, using the formula,

$$n = N - \frac{(H^+)}{\alpha'} \tag{119}$$

where n = the amount of acid or alkali bound;
N = the original normality of the acid or alkali;
(H^+) = the hydrogen ion concentration of the protein-acid solution at equilibrium;
α' = the degree of ionization of the acid as determined by potentiometric methods.

The assumption involved in this formula is that a given hydrogen ion concentration indicates a given normality of acid or alkali, irrespective of the presence or absence of protein or protein micelles in the system. They point out that this is probably not correct, but that since there was no method of calculating the effect of the protein on the equilibrium, it appeared to be more nearly correct than any other formula which had been proposed.

Later, Cohn criticized this formula and proposed a new formula which in turn involves several assumptions. The derivation of his formula is given in a consideration of the base-binding capacity of casein.[14] Using the fundamental equation (42), for deriving pH, *i.e.*,

$$\frac{\text{E.M.F. (observed)} - \text{E (calomel electrode)}}{0.00019837T} = \log \frac{1}{H^+} = p\text{H} \tag{120}$$

Cohn converts this into pOH by the equation,

$$p\text{H}^+ + p\text{OH}^- = p\text{K}_w \tag{121}$$

[14] Cohn, E. J., and Berggren, R. E. L., Studies in the Physical Chemistry of the Proteins. III. The Relation between the Amino Acid Composition of Casein and Its Capacity to Combine with Base, *J. Gen. Physiol.*, 7 : 45–79 (1924).

which in turn can be converted into the logarithmic expression,

$$p\mathrm{OH}^- = \log \frac{1}{(\mathrm{OH}^-)} \tag{122}$$

Cohn then introduces the activity coefficient (γ) in place of the dissociation coefficient (α') used by Hoffman and Gortner, or the (α) used by Lloyd and Mayes, the activity coefficient being determined by the ratio,

$$\frac{(\mathrm{OH}^-)}{(\mathrm{NaOH})} = \gamma \tag{123}$$

Combining equations (122 and (123), we have the equation,

$$p\mathrm{OH}^- = \log \frac{1}{(\mathrm{NaOH})} = p\mathrm{NaOH} + p\gamma \tag{124}$$

A number of workers have studied activity, and Lewis and Randall[15] have given values for the activity coefficient of the hydrogen and hydroxyl ions in various concentrations of hydrochloric acid and sodium hydroxide. Table LII shows the activity coefficients (γ) and the logarithms of the reciprocals of the activity coefficients ($p\gamma$) for varying concentrations of hydrochloric acid and sodium hydroxide.

From formula (124) Cohn calculates the logarithm of the reciprocal of the sodium hydroxide present in the sodium hydroxide solution to which protein has been added, and converts this into normality of sodium hydroxide. This value subtracted from the original normality, he regards as the amount of sodium which was bound by the protein. The assumptions involved in Cohn's method of calculation appear to the author to be more serious than the assumptions used by Hoffman and Gortner. In order to calculate the sodium hydroxide bound by Cohn's method, one must assume (*a*) that the sodium-protein compound is completely dissociated, and (*b*) that the sodium ions from the sodium-protein compound have the same activity as sodium ions in a sodium hydroxide solution and (*c*) that the protein ion does not influence this activity or rather that the influence of the protein ion on the activity of the sodium ion is the same as is the influence of an hydroxyl ion. Similarly, if Cohn's method were used to study acid-binding, one would have to assume the complete dissociation of the so-called " protein chloride " with the same activity coefficient for the chloride ions of the " protein chloride " as for the chloride ions in an equivalent concentration of hydrochloric acid. Various workers have indicated that either there is

[15] Lewis, G. N., and Randall, M., Thermodynamics and the Free Energy of Chemical Substances, McGraw-Hill Book Company, New York (1923).

TABLE LII

The Activity Coefficient (γ) (Lewis and Randall) and the Logarithms of the Reciprocal of the Activity Coefficient ($p\gamma$) for Various Concentrations of Hydrochloric Acid and Sodium Hydroxide.

Molarity	HCl		NaOH	
	Activity Coefficient	$\log \frac{1}{\gamma}$	Activity Coefficient	$\log \frac{1}{\gamma}$
	(γ)	($p\gamma$)	(γ)	($p\gamma$)
0.001	0.98	0.009	0.98	0.009
0.002	0.97	0.013	0.97	0.013
0.005	0.95	0.022	0.95	0.022
0.010	0.92	0.036	0.92	0.036
0.020	0.90	0.046	0.89	0.051
0.025	....	(0.048)	....	(0.055)
0.030	....	(0.050)	....	(0.059)
0.040	....	(0.053)	....	(0.065)
0.050	0.88	0.056	0.85	0.071
0.100	0.84	0.076	0.81	0.091

not complete dissociation of the " sodium proteinates " and " protein chlorides " or both anions and cations are adsorbed by the protein. If such a viewpoint is correct, Cohn's method of calculation introduces errors which may be as serious as the errors introduced by the formulae which have been previously employed.

Using the above formulae in his study of the base-binding capacity of casein, Cohn concludes that casein has a maximum base-binding capacity of approximately 0.0014 mol of sodium hydroxide per gram of casein which had never been exposed to greater alkalinities than those which exist in nature, whereas casein which has been prepared by more drastic treatment has a maximum base-binding capacity of 0.0018 mol of sodium hydroxide per gram, 1 mol of sodium hydroxide, therefore, combining with 735 grams of unaltered casein or with 535 grams of casein somewhat altered. He places the minimum molecular weight of casein as 12,800 and calculates that a molecule of this size contains 24 acid valences.

In Cohn's studies where he draws the conclusion that casein has a "maximum " base-binding capacity, he studied only concentrations of sodium hydroxide ranging between 0.03 N and 0.05 N. Hoffman and Gortner in their studies covered the range from 0.0005 N to 0.50 N. It seemed desirable, therefore, to repeat the work, using the technic and method of calculation exactly as suggested by Cohn. This has accord-

ingly been done by Gortner,[16] using the identical sample of casein employed in the studies by Hoffman and Gortner, as well as two other samples, both of which were prepared in such a manner that they were never exposed to a solution having a hydrogen ion concentration less than that represented by $p\mathrm{H} = 7.0$. In this study, Gortner found that the amount of sodium hydroxide which was bound increased progressively with increasing normality of sodium hydroxide which was employed, as shown in Table LIII, where a casein-sodium hydroxide system is compared with a succinic acid-sodium hydroxide system. It will be noted that the succinic acid-sodium hydroxide system reaches a maximum at the point where chemical binding should cease, and that the amount of base bound is not a function of the equilibrium hydrogen ion concentration. Table LIII substantiates the earlier publication of Hoffman and Gortner in their conclusion that the amount of base which is bound by casein is dependent upon the equilibrium hydrogen ion concentration, and does not confirm the statement of Cohn that casein possesses a maximum base-binding capacity.

Either the formula suggested by Cohn is inadequate for the calculation of base-binding, or the " sodium proteinate " is not completely dissociated at the higher concentrations of alkali, or sodium hydroxide is " adsorbed " on the ionic micelles according to a typical adsorption isotherm.

The author believes that casein does bind sodium hydroxide in the protein molecule by virtue of free acidic groups. His own studies and those of other workers lead him to believe, however, that adsorption processes play a role in increasing the amount of base or acid or salts which are drawn into the interfaces of the ionic micelles, and that we can ignore neither the forces of colloidal adsorption nor the forces of primary valence in a study of the physical chemistry of protein systems.

Thomas and Mayer[17] have used a novel method of estimating the acid-binding capacity of a protein. They have used a Zeiss-Löwe interferometer capable of measuring small changes in the refractive index of liquids. They find that the refractive indices of mixed solutions of gelatin and of hydrochloric acid are not additive, whereas the refraction of mixed solutions of " gelatin chloride " and of hydrochloric acid are additive. Accordingly, when a gelatin sol is titrated with an acid, there is an abrupt change in the slope of the curve at some particular acid concentration, which they believe indicates the amount of acid

[16] Unpublished data.

[17] Thomas, A. W., and Mayer, C. W., Estimation of the Acid Combining Capacity of a Protein by Means of the Interferometer, *Proc. Soc. Exp. Biol. Med.*, 25 : 667–669 (1928).

TABLE LIII

A Comparative Study of "Alkali Binding" by Succinic Acid and Casein* by Cohn's Method—(Data of Gortner)

Normality NaOH Used	Succinic Acid				Casein			
	Succinic Acid per Liter, Grams	Equilibrium p NaOH,	NaOH Bound, Normality	NaOH Bound per Gram, Equivalents	Casein per Liter, Grams	Equilibrium p NaOH	NaOH Bound, Normality	NaOH Bound per Gram, Equivalents
0.020	1.1804	6.649	0.02000	0.001694	10	2.509	0.01690	0.001690
0.040	1.1804	1.702	0.02012	0.001704	10	1.705	0.02028	0.002028
0.040	2.3608	5.641	0.03999	0.001694	20	2.419	0.03619	0.001809
0.050	1.1804	1.530	0.02048	0.001735	10	1.535	0.02083	0.002083
0.050	2.3608	2.008	0.04019	0.001702	20	1.961	0.03906	0.001953
0.060	.....	.....	.....	.....	10	1.412	0.02127	0.002127
0.060	.....	.....	.....	.....	20	1.701	0.04009	0.002005
0.060	.....	.....	.....	.....	30	2.332	0.05534	0.001845
0.075	.....	.....	.....	.....	10	1.282	0.02276	0.002276
0.075	2.3608	1.478	0.04170	0.001766	20	1.478	0.04173	0.002086
0.075	3.5412	1.780	0.05838	0.001649	30	1.810	0.05951	0.001984
0.100	1.1804	1.099	0.02038	0.001726	10	1.127	0.02536	0.002536
0.100	2.3608	1.226	0.04064	0.001721	20	1.268	0.04605	0.002302
0.100	3.5412	1.410	0.06110	0.001725	30	1.435	0.06327	0.002109
0.100	4.7216	1.739	0.08174	0.001731	40	1.721	0.08099	0.002025
			Average....	0.001713			Average....	0.002057
			Theory	0.001694				
			Maximum deviation from average	−3.7% / +3.1%			Maximum deviation from average	−17.8% / +24.2%

* Note the much greater variability of the casein values and that the alkali "bound" by casein increases with an increased equilibrium hydroxyl ion concentration.

which must be added to gelatin to form the " gelatin chloride." Their curve is shown in Fig. 111. The technic of Thomas and Mayer adds a new tool which should be of great value in studying the effects of acids and bases upon protein systems.

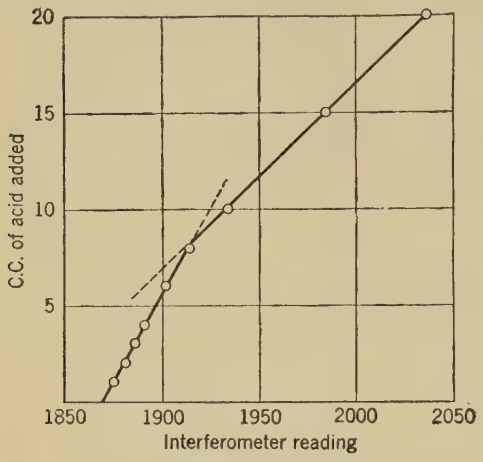


FIG. 111.—The titration curve of 0.881 grams gelatin with 0.1004 N HCl, volume 200 cc., as measured by readings in a liquid interferometer. (Data of Thomas and Mayer.)

Various other phases of the physico-chemical behavior of proteins could well be cited. Thus, for example, the optical rotation of proteins may change several hundred per cent at an altered hydrogen ion concentration,[18,19] or by peptizing protein sols with different neutral salts.[20] These observations of Carpenter are perhaps more surprising than the observations of Kraemer, in that Carpenter's solutions were adjusted to a *p*H of 6.0, and he nevertheless observed changes in the optical rotation of gelatin, ranging from $[\alpha]_D$ of $-340°$ to as low as $-105.5°$. The curves for KCl, KBr, and KI with increasing concentration are very different and indicate a striking lyotropic series similar to that already noted by Gortner, Hoffman, and Sinclair for the peptizing action of these salts.

For a more extended discussion of the physico-chemical properties of proteins in relation to the various environments, the reader must refer to Cohn's review which has already been noted, and to Robertson,[21] and to Pauli.[22]

[18] Kraemer, E. O., Observations of the Colloidal Behavior of Aqueous Gelatin Systems, Colloid Symposium Monograph, Vol. IV, pp. 102–121, Chemical Catalog Company, Inc., New York (1926).

[19] Fanselow, J. R., The Influence of Electrolytes and Non-Electrolytes upon the Optical Activity and Relative Resistance to Shear of Gelatin Systems, Colloid Symposium Monograph, Vol. VI, pp. 237–252 (1928).

[20] Carpenter, D. C., The Influence of Salts on the Optical Rotation of Gelatin. I., *J. Phys. Chem.*, 31 : 1873–1879 (1927).

[21] Robertson, T. B., The Physical Chemistry of the Proteins, Longmans, Green, and Company, New York (1918).

[22] Pauli, Wo., Colloid Chemistry of the Proteins, Pt. I, translated by P. C. Thorne, J. A. Churchill, London (1922).

CHAPTER XVIII

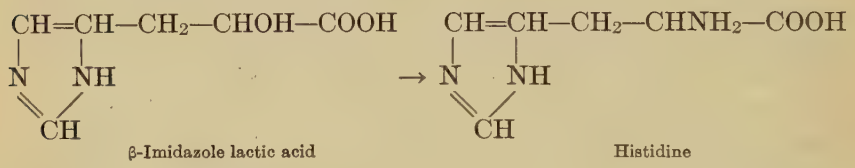
THE DIGESTION AND METABOLISM OF PROTEINS

Man (and, insofar as we know, every vertebrate) is absolutely dependent upon the plant kingdom for the amino acids which he needs to synthesize into the proteins characteristic of his own tissues. Whether or not forms of animal life below the vertebrates are similarly dependent is still an open question, and there has been little or no investigational work in this field. *The vast majority of the amino acid molecules in our bodies are derived from plant proteins where they are produced from their inorganic constitutents by the action of photosynthesis.* To be sure some of the amino acids in the proteins of our bodies may have been derived from the proteins of animals used by us as foods, but the proteins of these animals, in the last analysis, were composed of amino acids obtained from plants, and apparently they have been passed down to us unchanged in their chemical configuration.

Glycine appears to be the only amino acid that we or other vertebrates can synthesize in our bodies, at least in adequate amounts for growth or maintenance. Whether or not all of the other amino acids must be secured solely from our sources of protein foods is still an open question. *We know that cystine, histidine, tryptophane, and lysine are essential, that probably tyrosine or phenylalanine, arginine and glutamic acid are essential, and that possibly proline is essential, and that vertebrates cannot synthesize these amino acids.* It will be noted that these amino acids are either readily isolated or have some characteristic reaction by which they can be readily identified. Accordingly we can determine whether or not they are present in the foodstuffs which are eaten, and we can likewise determine the approximate quantity which is consumed. The difficulty in proving that certain of the other amino acids, not included in the above list, are or are not essential lies in the difficulty in securing foodstuffs from which we can prove that they are absent. Our analytical methods are too crude to prove that they are completely absent, and synthetic mixtures of amino acids are too expensive for the necessary feeding experiments, using such mixtures to replace protein in the diet.

The utter dependency of the vertebrates upon the plant kingdom per-

haps cannot be better expressed than by the statement that the vertebrate cannot even insert an $—NH_2$ group into the ϵ-carbon atom of α-amino caproic acid to form α-ϵ-diamino caproic acid or lysine, this in spite of the fact that the animals were starving to death because there was no lysine in the diet, and were deliberately given an adequate amount of α-amino caproic acid (cf. Lewis and Root[1]). Similar experiments have been conducted with other synthetic materials with essentially similar results, except that Cox and Rose[2] report that *d-l*-β-4-imidazole lactic acid, when fed with diets deficient in histidine, caused a resumption of growth, indicating that an amino group had apparently been inserted in place of the —OH group of the lactic acid radical. This observation has been confirmed by Harrow and Sherwin,[3] who further show that imidazole pyruvic acid is able to some extent to replace histidine in the diet. The reaction is apparently,



although no proof other than the resumption of growth is given for the synthesis of histidine. It may be that no amino group is inserted in the substituted imidazole, that the β-imidazole lactic acid, *as such*, functions in place of the histidine.

Methyl imidazole, hydroxymethyl imidazole, imidazole carboxylic acid, β-imidazole propionic acid, and β-imidazole acrylic acid did not supplement histidine-deficient diets.

The modern ideas of protein nutrition[4] are vastly different from those annunciated by Liebig. The old idea involved the presence of an ample supply of protein, carbohydrate, fat, and mineral matter. Later, physiologists introduced the qualification that a minimal amount of energy was required, and accordingly the calorific equivalent of the diet was added. The newer viewpoint, largely brought about by the researches of Osborne and Mendel, and McCollum and his coworkers, has shown

[1] Lewis, H. B., and Root, L. E., Amino-Acid Synthesis in Animal Organism. Can Nor-Leucine Replace Lysine for the Nutritive Requirements of the White Rat, *J. Biol. Chem.*, 43 : 79–87 (1920).

[2] Cox, G. J., and Rose, W. C., The Availability of Synthetic Imidazoles in Supplementing Diets Deficient in Histidine, *J. Biol. Chem.*, 68 : 781–799 (1926).

[3] Harrow, B., and Sherwin, C. P., Synthesis of Amino Acids in the Animal Body. IV. Synthesis of Histidine, *J. Biol. Chem.*, 70 : 683–695 (1926).

[4] McCollum, E. V., and Simmonds, Nina, The Newer Knowledge of Nutrition, Third Edition, The Macmillan Company, New York (1925).

that *protein quantity* is not an adequate criterion of diet, and that *protein quality* must be taken into consideration, *i.e.*, the question must be answered, " Does the protein which is eaten contain the essential amino acids in adequate amounts for normal growth and maintenance?" To use Dr. McCollum's illustration, if the body needs " Peter-Piper-picked-a-peck-of-pickled-peppers " protein, it will not secure normal nutrition but will starve to death on a " Theosophilus-Thistle-the-successful-thistle-sifter " protein.

We cannot go into the role played by the individual amino acids, nor into the experiments which prove them to be essential. Any modern text on nutrition will be found to cover these points.

All that can be emphasized is the striking specificity of the biological processes involved both in growth and maintenance, as illustrated by the fact that if one essential amino acid is lacking from the diet, the entire growth mechanism may break down completely and the animal starve to death.

The whole viewpoint of the mechanism of protein metabolism within the animal body has changed within the last few years almost as radically as have the ideas of what constitutes normal nutrition. The older workers supported the view that the proteins were digested by pepsin and hydrochloric acid in the stomach to proteoses and peptones and that these were further digested to peptides and amino acids by the tryptic and ereptic enzymes of the duodenum and small intestine. It was then believed that the peptides were absorbed, as such, through the intestinal wall and were resynthesized into protein in the intestinal wall, passing into and being transported by the blood stream as proteins.

Van Slyke and Meyer[5] report the presence of *amino nitrogen* in the non-protein nitrogen fraction of the blood, and that the non-protein amino nitrogen in the blood increased following a meat diet, thus indicating that free amino acids were transported, as such, in the blood stream. However, the presence of amino nitrogen did not definitely prove the presence of the simple amino acids, for it may have been derived from relatively simple peptides or from other compounds.

We have already noted the vividiffusion apparatus of Abel, and insofar as the author is aware, Abel should be credited with the first absolute proof that amino acids are transported, as such, in the blood stream. Shortly following the preliminary publication of Abel,[6]

[5] Van Slyke, D. D., and Meyer, G. M., The Amino-Acid Nitrogen of the Blood. Preliminary Experiments on Protein Assimilation, *J. Biol. Chem.*, 12 : 399–410 (1912).

[6] Abel, J. J., Rowntree, L. G., and Turner, B. B., On the Removal of Diffusible Substances from the Circulating Blood by Means of Dialysis, *Trans. Assoc. Am. Physicians*, 28 : 51–54 (1913).

Abderhalden[7] reported the isolation of a considerable quantity of amino acids from blood, thus confirming Abel's observations by an independent method.

These series of observations changed the earlier theories to the modern belief that the amino acids are transported as such in the blood stream to the various cells and tissues, where such as are needed are resynthesized into the proteins characteristic of those cells and tissues.

Gastric digestion by pepsin reduces proteins only to the stage of proteoses and peptones. Probably no simple amino acids are formed by gastric digestion and probably no appreciable absorption takes place from the stomach. We can, therefore, look upon gastric digestion of protein as a preliminary hydrolysis and hydration, rendering the split products more susceptible to enzyme action after the food has passed into the duodenum.

In intestinal digestion, the proteins are attacked by trypsin in the upper portion of the intestines and by erepsin somewhat lower down. Both trypsin and erepsin hydrolyze proteins to their constituent amino acids. These amino acids then diffuse through the intestinal wall into the blood stream, and, as already noted, are transported to the various cells and tissues of the body. At any one time there is an extremely small concentration of amino acids in the blood stream. It has been calculated that at the height of digestion the rate of blood flow through the portal vein in a 9.5 kilogram dog is approximately 9000 cc. per hour, or at a rate of 150 cc. per minute. Protein is only slowly digested and absorbed. Pflüger has stated that the absorption of 1.14 grams of protein per kilo of body weight per hour is a good absorption for a human being. Accordingly if such an absorption of protein were to take place in the dog noted above, the concentration of amino acids in the blood would not exceed 0.12 per cent. Some workers place this value as low as 0.005 per cent.

It is not surprising therefore that by the earlier and somewhat crude methods, the presence of amino acids in the blood stream was overlooked and that only the more refined technic of the vividiffusion or the Van Slyke apparatus was able to detect the presence of appreciable quantities of amino acids. Abderhalden in his isolation of amino acids from the blood stream worked up approximately 100 liters of blood and identified proline, leucine, valine, aspartic and glutamic acids, glycine, arginine, histidine, and lysine. No amino acid was found in amounts exceeding 0.40 gram.

[7] Abderhalden, E., Der Nachweis von freien Aminosäuren im Blute unter normalen Verhältnissen, *Z. physiol. Chem.*, 88 : 478–483 (1913).

It should be noted at this point that the older workers were justified in concluding that amino acids were not present, partly because of the inadequacy of their methods and partly because of the fact that certain other nitrogenous compounds, for example, urea, creatine, creatinine, uric acid, ammonia, etc., are always present in the blood serum from which the proteins have been removed by appropriate technic. Some of these constituents may well occur in much larger quantities than the amino acids, and accordingly unless very delicate technic were employed, it would be impossible to identify such traces of amino acids as are normally present during the absorption of protein.

The question is perhaps pertinent as to whether or not polypeptides, proteoses, peptones, and perhaps even proteins may not at times pass from the digestive tract into the circulation. The author believes that this is not only a possibility but a probability. Certain individuals and particularly infants develop pathological conditions which can be traced to the inclusion of some particular protein in the diet, in that they become exceedingly hypersensitive to some of these proteins. Egg albumin appears to be one of the most common proteins producing such hypersensitivity, although many cases have been noted where the proteins of the legumes, particularly peas, are involved. We have already noted that the egg albumin molecules are so small that they will dialyze slowly through a fairly porous collodion membrane. It would seem probable, therefore, that conditions may arise whereby the membranes separating the intestinal contents from the blood stream become sufficiently permeable to permit the passage of small amounts of foreign proteins, thus giving rise to protein intoxication. Aside from its pathological significance this does not appear to be an important problem in protein assimilation.

No adequate theory has been advanced to account for the synthesis of the vital proteins in the various cells and tissues from the heterogeneous mixture of amino acids which are transported in the blood stream. We only know that the vital process involved is one of rigid selection and that a given cell or tissue invariably synthesizes proteins containing certain specific amino acids linked in a certain specific way. We have no clue as to the mechanism whereby this selective action is brought about.

The Degradation of Amino Acids in the Body.—The body apparently possesses a very efficient mechanism for maintaining a low concentration of amino acids in the blood stream even when protein digestion and absorption are at a maximum. This mechanism appears to reside chiefly in the liver where the amino acids are deaminized and broken down, the nitrogenous portion being eliminated as urea by the kidneys.

Underhill[8] has discussed certain phases of this problem in an excellent manner.

Deamination may occur due to several different reactions. Thus, *simple deamination* might yield a ketonic aldehyde,

$$R—CH(NH_2)COOH \rightarrow R—CO—CHO + NH_3,$$

or the reaction may involve the elements of water and result in a *hydrolytic deamination,* with the formation, as the primary product, of an α-hydroxy acid.

$$R—CH(NH_2)COOH + H_2O \rightarrow R—C(OH)—COOH + NH_3.$$

Armsby favors the hydrolytic deamination, for he notes that the reaction is essentially isothermic, a characteristic of hydrolytic processes. The metabolism of an amino acid may be further represented as that of an *oxidative deamination* which can be represented as follows:

$$R—CH_2—CH(NH_2)COOH + O = R—CH_2—COOH + NH_3 + CO_2,$$

the carbon dioxide and ammonia being reunited to form urea. Under certain conditions, instead of forming a saturated acid, the oxidative deamination yields an α-ketonic acid, or the first stage may be the formation of a hydroxy amino acid which undergoes deamination to form the corresponding ketonic acid, and this by loss of carbon dioxide forms a fatty acid containing one less carbon than the original amino acid.

$$R—CH_2—CH(NH_2)COOH + O = R—CH_2—CO—COOH + NH_3$$

or

$$R—CH_2—CH(NH_2)COOH + O = R—CH_2—COH(NH_2)COOH \rightarrow R—CH_2—CO—COOH + NH_3$$

$$R—CH_2—CO—COOH = R—CH_2—COOH + CO_2$$

Occasionally under pathological conditions, the oxidation of amino acids does not follow the normal process. In such cases we have the formation not of α-ketonic acids but of β-ketonic acids. These are very resistant to further oxidation. Such types of oxidation are characteristic of diabetes where appreciable quantities of β-oxy butyric acid and of aceto-acetic acids may be formed. The presence of any appreciable

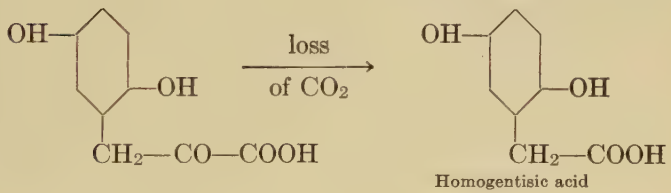
[8] Underhill, F. P., The Physiology of the Amino Acids, Yale University Press, New Haven (1915). *Cf.* also Mitchell, H. H. and Hamilton, T. S., The Biochemistry of the Amino Acids, Chemical Catalog Co., Inc., New York (1929).

quantities of these β-ketonic acids (derived from either protein or fat) are indicative of a serious pathological condition.

The amino acids containing an aromatic nucleus apparently undergo the α-ketonic oxidation, and there is some evidence that a part of the aromatic nuclei may be opened and burned within the animal body. However, the phenols which are normal constituents of the urine, and indole, skatole, indigo blue, etc., appear to have their origin in the benzene and indole rings of the amino acids by action of the intestinal bacteria.

Under certain conditions the mode of oxidation of certain of the amino acids appears to be altered within the animal body, tyrosine, for example, giving rise to homogentisic acid, probably according to the following scheme:

$$OH(p)C_6H_4{-}CH_2{-}CHNH_2{-}COOH \xrightarrow[\text{deaminize}]{\text{oxidize and}} OHC_6H_4{-}CH_2{-}CO{-}COOH \xrightarrow[\text{rearrange}]{\text{oxidize and}}$$



Homogentisic acid

The homogentisic acid appears in the urine in certain diseases, such as melanouria and alkaptonuria, the urine being colorless when voided and rapidly darkening to an intense black liquid from which black particles are precipitated.

The presence of homogentisic acid in the urine should not be taken necessarily as an indication of a pathological condition. This mechanism for the oxidation of tyrosine and phenylalanine appears to be hereditary, is more prevalent in males than in females, and may persist throughout life without any evidence of harmful effects. An increased consumption of proteins containing tyrosine or phenylalanine will increase the amount of homogentisic acid which is excreted.

The sulfur of cystine on oxidation in the body yields sulfuric acid and sulfates. Again, in this instance, cystineuria may occur, the cystine being eliminated as such in the urine. This again need not necessarily be

taken as indication of a pathological condition, inasmuch as it is hereditary, affecting males and females about equally. The only untoward symptom which may arise is the aggregation of the cystine crystals into kidney stones or urinary concretions. Cystine is so insoluble that there are a number of instances on record where such concretions have been formed. Thus, Tennant[9] reports a surgical case in which 15 stones, having a total weight of 73 grams, were removed from a kidney. Gortner and Hoffman[10] report that these stones contained 93 per cent of pure cystine.

The Formation of Carbohydrates and Fats from Amino Acids.—Carbohydrates and fats may be formed from proteins under the normal processes of metabolism. In studies as to the factors influencing sugar excretion by diabetics, it had long been suspected that protein might give rise to carbohydrates.

Lusk administered amino acids to phlorhizinized dogs and found that certain of the amino acids yielded sugar, whereas others did not. Those containing 2, 3, 4, and 5 carbon atoms in a straight chain were more or less completely converted into glucose. Glycine and alanine could be quantitatively converted into glucose, whereas only three of the carbon atoms of aspartic and glutamic acids were converted into glucose, three-fourths of the carbon of the aspartic acid and three-fifths of the carbon of the glutamic acid appearing in the form of glucose. Lusk believes that the intermediate products are either glycolic acid, lactic acid, or glyceric acid, depending on the amino acid involved. Thus three molecules of glycine on deamination yield three molecules of glycolic acid which are reduced to three molecules of glycolic aldehyde, which in turn condense to one molecule of glucose.

$$\begin{array}{l} CH_2{-}NH_2 \\ | \\ COOH \end{array} \longrightarrow \begin{array}{l} CH_2OH \\ | \\ COOH \end{array} \longrightarrow \begin{array}{l} CH_2OH \\ | \\ CHO \end{array} \longrightarrow C_6H_{12}O_6$$

And two molecules of alanine will yield two molecules of lactic acid which condense to form one molecule of glucose, or aspartic acid may yield β-lactic acid and carbon dioxide, two molecules of the lactic acid condensing to form glucose. Again glutamic acid may yield glyceric acid and acetic acid, the glyceric acid being transformed into glyceric aldehyde, two molecules of which condense to form one molecule of glucose.

Dakin has shown that serine, proline, arginine, and ornithine will

[9] Tennant, C. E., Cystin Calculi: A Complex Surgical Problem, *J. Am. Med. Assoc.*, 80 : 305–307 (1923).

[10] Gortner, R. A., and Hoffman, W. F., A Chemical Study of Cystine from Kidney Stones, *Proc. Soc. Exp. Biol. Med.*, 23 : 691–693 (1926).

yield at least a part of their carbon in the form of glucose, the arginine going first to ornithine. Lysine is the only straight-chain amino acid tested, which failed to yield sugar.

The Specific Dynamic Action of Proteins.—One additional property of proteins and amino acids in metabolism deserves mention, *i.e.*, the phenomenon which Rubner called the specific dynamic action of proteins. It had been noted by many workers that increased metabolism, as measured in a respiration calorimeter, resulted when proteins were fed. If an amount of sugar equivalent to a given number of calories is fed to a fasting dog, the heat, as measured in a respiration calorimeter, is slightly lower than the heat which would be produced by the combustion of the sugar which was fed. If, however, protein having an equivalent calorific value, replaces the sugar, then the heat, as measured in a respiration calorimeter, exceeds by approximately 5 per cent the calorific value of the protein which was fed.

This phenomenon of excess heat production following the feeding of protein and of amino acids has been extensively investigated by Lusk and Benedict who have shown that the excess heat is not due to mechanical heat from increased intestinal activity or peristaltic action. Lusk has suggested that the amino acids behave as cell stimulants, raising the metabolic power of the tissue cells, the excess energy being derived from carbohydrates and fats which are burned in amounts greater than the normal level. Lusk[11, 12] suggests that perhaps the stimulant is the keto or the oxy-acids formed as intermediate metabolic products.

It should be noted in passing that the amino acid, thyroxine, isolated by Kendall from the protein of the thyroid gland apparently regulates in a large measure the metabolism of the body. Apparently other amino acids reflect in a small measure the extraordinary effects of thyroxine.

Enzymatic Synthesis of Proteins.—Robertson[13] discusses the earlier literature in regard to the enzymatic synthesis of proteins. Bourquelot (*vide infra*) has shown that the reaction between glucosides and glucoside-splitting enzymes is a reversible reaction according to the following scheme:

$$\text{Glucoside} + \text{enzyme} \rightleftharpoons \text{enzyme} + \text{sugar} + x,$$

where x represents a non-sugar radical. Accordingly, in dilute solutions

[11] Lusk, Graham, The Elements of the Science of Nutrition, Third Edition, W. B. Saunders Company, Philadelphia (1917).

[12] Lusk, Graham, Problems of Metabolism, Mayo Foundation Lectures on Nutrition, pp. 59–75, W. B. Saunders Company, Philadelphia (1925).

[13] Robertson, T. B., The Physical Chemistry of the Proteins, Chapter XVII, Longmans, Green and Company, New York (1918).

the reaction is driven toward the right, resulting in the hydrolysis of the glucoside, whereas in concentrated solutions of the components plus the enzyme, the reaction is driven toward the left and the glucoside is resynthesized.

The experiments carried out by Robertson and his coworkers indicated that a similar equilibrium occurred in the case of protein and proteolytic enzymes.

Taylor[14] digested the protamine, salmin, with trypsin, converted the amino acids into carbonates, destroyed the trypsin by heat, and concentrated the amino acids from 400 grams of the protamine to the point of crystallization. He then added 300 cc. of the glycerol extract of trypsin from the liver of a clam, added toluene as a preservative, and set the mixture aside. The mixture gradually became opalescent, then cloudy, and finally a precipitate formed. At the end of five months, 2 grams of salmin sulfate was isolated from the mixture. A duplicate mixture, omitting the enzyme, yielded no trace of the protamine. Robertson[15] similarly reports the synthesis of " paranuclein " from the hydrolytic products of casein in the presence of pepsin.

Recently this problem has been attacked by Wasteneys and Borsook[16, 17] and the conclusion is reached that proteins may be synthesized by enzymatic action, probably as the result of an equilibrium similar to that noted above for the glucosides.

The last paper which is cited is of particular interest, in that the synthesis was carried out in the presence of emulsions, thus providing an increased surface area and accordingly an increased surface energy in the system. Wasteneys and Borsook note that the proteins which are synthesized differ from each other, depending upon the mixture of amino acids to which the enzyme has been added.

Whether or not the mechanism of the synthesis of proteins within the animal body is similar to the mechanism used in these *in vitro* experiments, is still an open question. In all of the experiments which have

[14] Taylor, A. E., On the Synthesis of Protein through the Action of Trypsin, *J. Biol. Chem.*, 3 : 87–94 (1907).

[15] Robertson, T. B., Note on the Synthesis of a Protein through the Action of Pepsin, *J. Biol. Chem.*, 3 : 94–99 (1907).

[16] Wasteneys, H., and Borsook, H., The Enzymatic Synthesis of Protein. I. The Synthesizing Action of Pepsin, *J. Biol. Chem.*, 62 : 15–29 (1924); II. The Effect of Temperature on the Synthesizing Action of Pepsin, *J. Biol. Chem.*, 62 : 633–639 (1925); III. The Effect of the Hydrogen ion Concentration on Peptic Synthesis, *J. Biol. Chem.*, 62 : 675–686 (1925).

[17] Wasteneys, H., and Borsook, H., The Effect of Emulsification in the Peptic Synthesis of Protein, Colloid Symposium Monograph, Vol. VI, pp. 155–172, Chemical Catalog Company, Inc., New York (1928).

been recorded the concentration of amino acids is much higher than any concentration known to occur within the tissues. However, it seems highly probable that the synthesis within the tissues may be brought about by a similar mechanism and that an effective high concentration of amino acids may result from the " binding " of a large part of the water within the cell, thus effectively concentrating the amino acids to a point where synthesis in the presence of proteolytic enzymes takes place.

CHAPTER XIX

THE BIOLOGICAL REACTIONS OF THE PROTEINS

No discussion of the importance of the proteins in biological processes would be complete without at least a casual mention of the important role which they play in the problems of immunity. Wells[1, 2] has summarized the more important literature with particular reference to the chemical problems which are involved. Accordingly, in the following pages only a very brief outline of these questions will be considered.

When foreign proteins are injected into a living animal, the injection being subcutaneous, intraperitoneal, or intravenous, they give rise to the formation of specific substances in the blood serum of the animal, the presence of which may be detected by subsequent reactions of the animal or the blood serum of the animal. The reactions which follow the injection of foreign proteins may be classified into four groups, (1) anaphylaxis, (2) the precipitin reaction, (3) hemolysis, and (4) complement fixation.

1. Anaphylaxis.—When a small quantity of a protein (a *sensitizing* dose) is injected into the blood stream of an animal and an appropriate time interval (7 to 30 days or more, depending upon the initial dosage, a larger dosage requiring a longer time) is allowed to elapse, a second injection of the same protein (the *intoxicating* dose) will cause the animal to undergo a severe shock, death often ensuing within a few minutes. The physiological reaction which occurs is known as the anaphylactic shock. The amount of protein necessary for the sensitizing dose may be exceedingly small, as little as 0.000,000,05 gram of egg albumin being sufficient. The initial injection has sensitized the animal to this particular foreign protein. The second injection brings about the anaphylactic reaction.

It seems probable that " hay fever " represents a sensitized condition of the individual to the proteins of certain pollens, and in many instances

[1] Wells, H. Gideon, The Chemical Aspects of Immunity, Chemical Catalog Company, Inc., New York (1925).

[2] Wells, H. Gideon, The Chemical Basis of Immunological Specificity, *J. Immunology*, 9 : 291–307 (1924).

eczema, particularly that of infants, and infant " colic " are due to a hypersensitivity to some particular protein.

2. The Precipitin Reaction.—When an initial injection of a foreign protein into an animal is followed by other injections at three- to four-day intervals, gradually increasing the dosage until an appreciable quantity, 0.25 gram or more of the foreign protein, has been injected in a series of from 5 to 6 up to 20 or 30 injections, the blood serum of the animal acquires the property of precipitating the particular foreign protein which was injected, when the immune serum is added to a solution of that protein *in vitro*. The delicacy of the test depends somewhat upon the nature of the protein injected and upon the potency of the immune sera used. The test is often used to detect human blood in criminal cases. Ordinary chemical tests for blood have a sensitivity which will detect dilutions of blood not much greater than 1 : 1000. The precipitin reaction has been found to be positive at a dilution of 1 : 50,000 for blood, and at a dilution of 1 : 1,000,000 for egg albumin. The " agglutination " reactions of bacteria are probably due to the same mechanism as the precipitin reaction of proteins.

3. Hemolysis.—When red blood cells of an animal are injected into the blood stream of an animal of a different species through a series of rather shortly spaced injections, the blood serum of the injected animal acquires the ability to dissolve the foreign blood corpuscles when tests are made *in vitro*. Here again, the reaction has been employed in criminal cases to ascertain whether or not the cells in a blood stain were human corpuscles. Similarly, " bacteriolysins " may be formed, which will disintegrate specific bacteria. Natural or acquired immunity may at least in part be due to the presence of such bacteriolysins.

4. Complement Fixation.—This reaction depends upon the fact that immune sera contain at least two distinct substances, both of which are required for the production of an immuno-reaction. These are (*a*) the relatively heat-stable " antibody," and (*b*) the complement which is destroyed at 55° C. Complement occurs in fresh, normal serum and can be supplied from that source.

Five biological reagents are necessary to carry out the complement fixation test.

Reagent (1) is a suspension of red blood cells (*e.g.*, sheep cells).

Reagent (2) is the immune serum (A) (*e.g.*, the serum of a patient who is suspected of having typhoid fever). The complement of this immune serum has been previously destroyed by heating the serum to 55° C.

Reagent (3) is a supply of fresh, normal serum to act as a source of complement.

Reagent (4) is the hemolytic serum (B) (*e.g.*, serum from rabbits which have been immunized to sheep corpuscles). This hemolytic serum (B) has had the complement destroyed by heating to 55° C.

Reagent (5) is a suspension of the bacteria causing typhoid fever.

The test is carried out by taking the immune serum (A)[reagent (2)], adding complement [reagent (3)], and then adding the bacteria suspension [*the antigen*, reagent (5)]. If reagent (2) is in reality a typhoid immune serum, combination will occur between the typhoid bacteria, the typhoid antibody, and the complement [reaction (I)], which will remove all of the complement from the solution and " fix " (adsorb ?) it on the agglutinated or precipitated bacteria. However, it may not be possible for us to detect this reaction. It is necessary, accordingly, to determine whether or not the reaction (I) has taken place. It is here that reagents (1) and (4) are used. To the original mixture of typhoid bacteria, typhoid antibody and complement are now added reagents (1) and (4). *If hemolysis occurs, reaction* (I) *did not take place*, and complement is still present in the solution, as evidenced by the fact that the red cells were broken down by the hemolytic serum (B). *If hemolysis does not take place, reaction* (I) *has already occurred, and reagent (2) was in reality serum from a patient with typhoid fever*, all the complement having been used up in reaction (I).

A modification of this reaction, but essentially the same insofar as the technic is concerned, is the Wassermann test[3] for syphilis.

Are the Immuno-Reactions Specific for a Particular Protein? —Wells (note particularly *loc. cit.* pages 63–85) considers this question at length and points out that *specificity depends upon the chemical structure of the protein molecule and not necessarily upon the biological origin of the protein*, although in the great majority of instances proteins of different species are different both chemically and immunologically.

Wells has, however, observed that the globulin from the seeds of the cantaloupe is immunologically identical with the globulin from squash seeds and that the caseins from the milk of animals of different species show very close biological relationships. The same is true of the vitellins of the egg yolk from various species of animals. Wells notes that " casein from the milk of an animal of any given species shows a closer biologic relationship to the casein of another species than it does to either the whey proteins or the serum proteins of its own species," and " egg yolk proteins from even such widely different species as fish and turtle may give precipitin reactions with the antiserum for hen egg yolk proteins."

[3] Noguchi, H., Serum Diagnosis of Syphilis, Third Edition, J. B. Lippincott Company, Philadelphia (1912).

Wells points out that phylogenetic relationships may be traced by the use of immunological reactions. A number of workers have busied themselves in this field. Thus Magnus[4, 5] and Zade[6, 7] have studied some of the relationships existing between plants and have constructed a " family tree " for certain of the leguminosae and gramineae. Nuttall[8] has studied more than 16,000 precipitin reactions quantitatively with the blood of more than 900 species of animals and found that antihuman precipitating serum gave comparative volumes of precipitate when tested against the blood sera of various orders of primates, as shown in Table LIV. Landsteiner and Miller[9] have added to these observations.

TABLE LIV

Showing the Intensity of the Precipitin Reaction between the Serum of an Animal Immunized against Human Blood Proteins and Equivalent Amounts of Blood Sera from Various Orders of Primates.

Blood Serum from	Number of Individuals Tested	Intensity* of Precipitin Reaction
Human	34	100
Simiidae (Anthropoids)	8 (3 species)	100
Cercopithecidæ (Common monkeys of the old world)	36	92
Cebidae (Capuchins and spider monkeys of the new world)	13	78
Hapalidae (Marmosets)	4	50
Lemuridae (Lemurs)	2	0

* 100 indicates a reaction essentially identical in intensity with that of the original serum used for immunizing.

We have already noted that Lewis and Wells found immunological relationships between the prolamines of *Triticum vulgare*, *T. dicoccum*, *T. monococcum*, and *T. spelta*. They similarly found immunological

[4] Magnus, W., Die Erkennung von Mehlverfälschungen durch die serumdiagnostische Methode, *Landw. Jahrb.*, 38 (Erg. bd. V) : 207–215 (1909).

[5] Magnus, W., Weitere Ergebnisse der Serum-Diagnostik für die theoretische und angewandte Botanik, *Ber. Bot. Ges.*, 26a : 532–539 (1908).

[6] Zade, A., Serologische Studien an Leguminosen und Gramineen, *Z. Pflanzenzüchtung*, 2 : 101–151 (1914).

[7] Zade, A., Die Verwendbarkeit der Präzipitinreaktion in der Samenprüfung, *Fühlings landw. Ztg.*, 61 : 807–810 (1912).

[8] Nuttall, G. H. F., Blood Immunity and Blood Relationship, Cambridge University Press, (1904).

[9] Landsteiner, K., and Miller, C. P., Jr., Serological Observations on the Relationship of the Bloods of Man and the Anthropoid Apes, *Science*, 61 : 492–493 (1925).

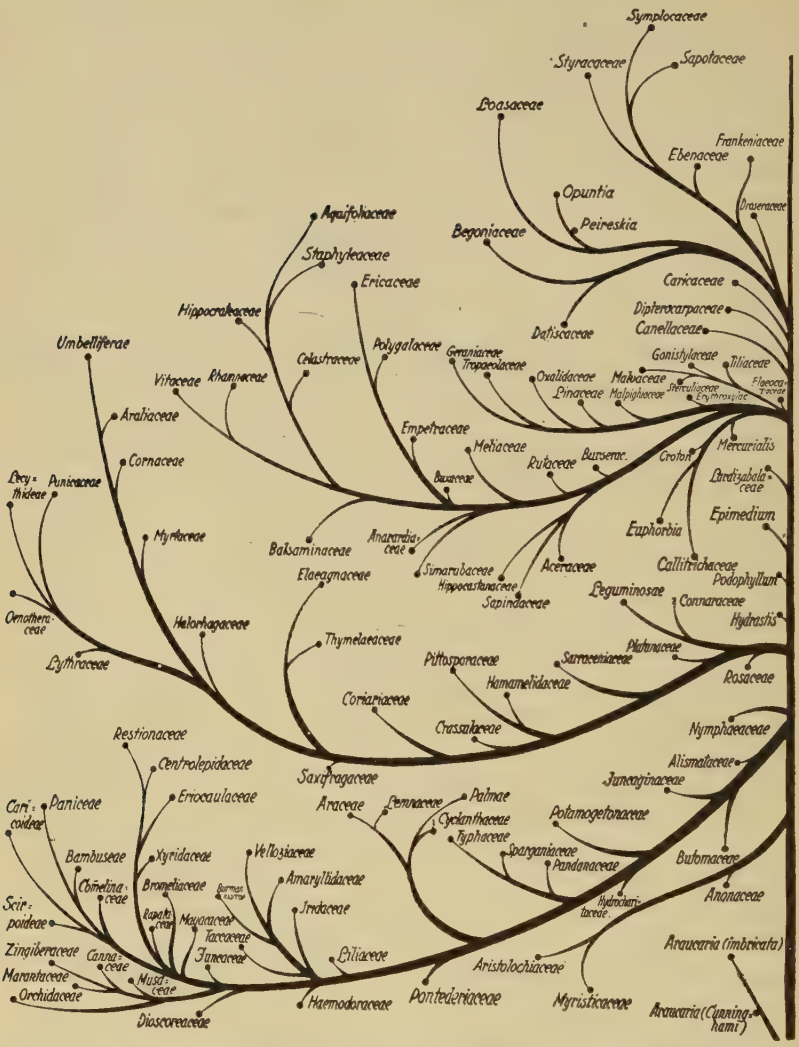


FIG. 112A.—The phylogenetic relations of plant species and genera, as indicated by serologic reactions. (Data of Mez.) Reproduced by permission of Dr. Carl Mez and the *Botanischen Archivs.*

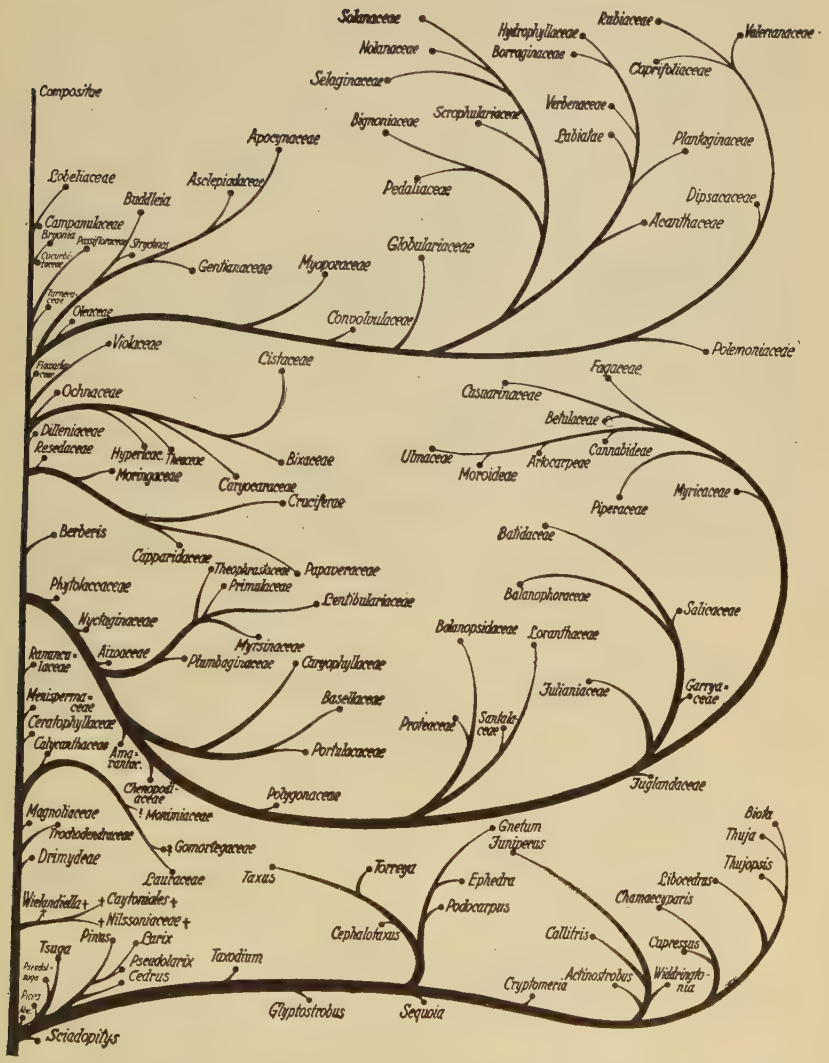


FIG. 112B.—The phylogenetic relations of plant species and genera, as indicated by serologic reactions. (Data of Mez.) Reproduced by permission of Dr. Carl Mez and the *Botanischen Archivs.*

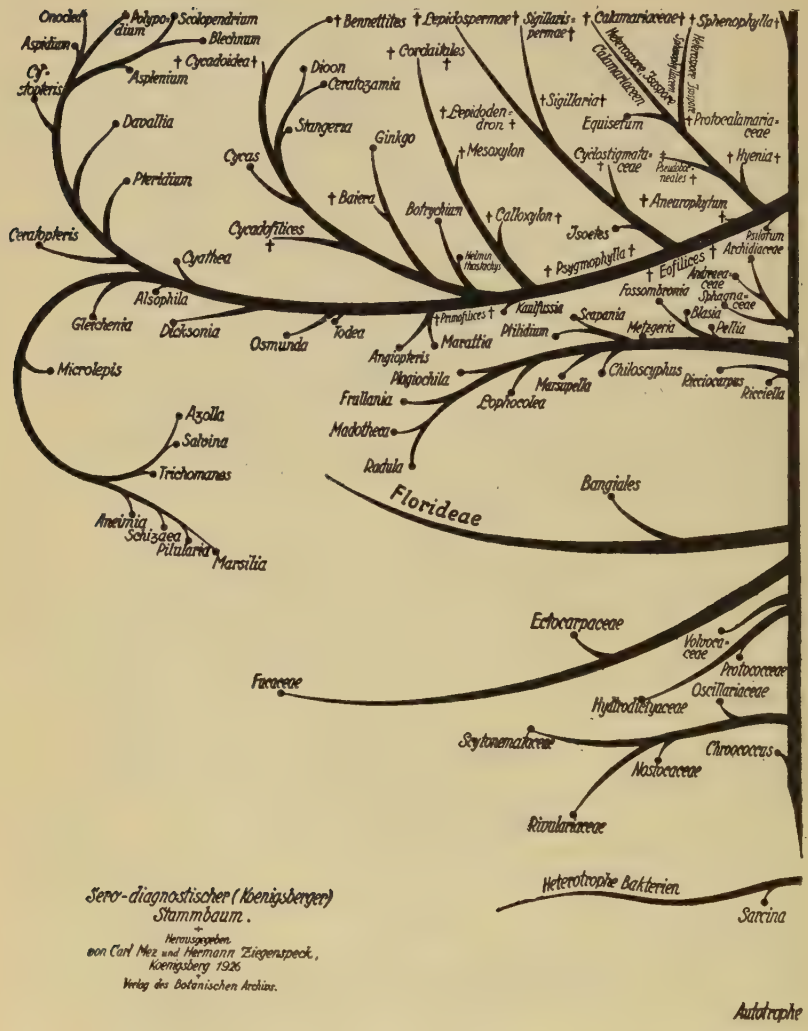


FIG. 112C.—The phylogenetic relations of plant species and genera, as indicated by serologic reactions. (Data of Mez.) Reproduced by permission of Dr. Carl Mez and the *Botanischen Archivs*.

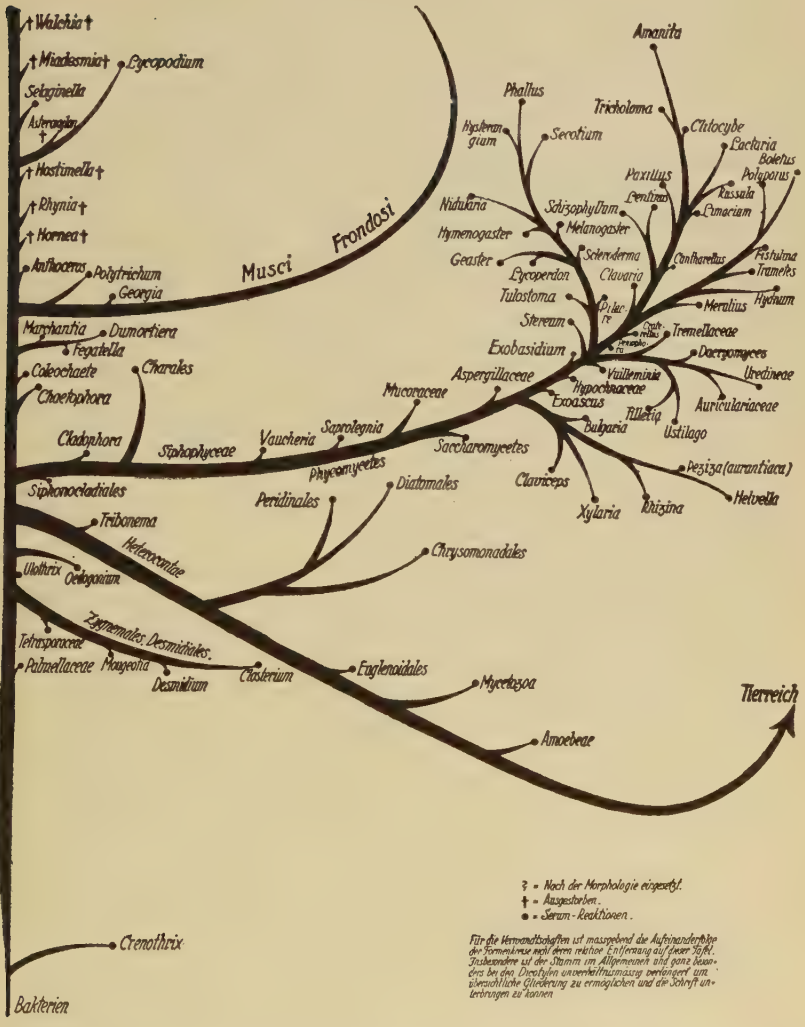


FIG. 112D.—The phylogenetic relations of plant species and genera, as indicated by serologic reactions. (Data of Mez.) Reproduced by permission of Dr. Carl Mez and the *Botanischen Archivs*.

relationships between the prolamines of the corn group, *Zea mays*, *Andropogon sorghum*, and *Euchlaena mexicana* Schrad, but no relationship between the " wheat " group and the " corn " group.

Perhaps the most extensive series of immunological studies are those conducted by Mez[10] and his coworkers. Mez[11] reports the technic which has been used in his laboratory for securing immune sera. He has used the proteins of the plant tissues and believes that his reactions are characteristic of the nucleoproteins, these carrying the hereditary factors of the plants investigated. All of the plant products used in his laboratory were given numbers and all injections and immunological reactions referred to a given material as material having a certain number. At the conclusion of a series of tests, the reactions of a given immune serum with a series of proteins derived from different plants were graded in the order of their intensity and recorded on a large chart by plant number. Lines or circles were then drawn through the plant numbers having the same intensity of reaction toward a given antiserum. After such a chart had been constructed, the botanical names were inserted in place of the material number, resulting in a " family tree " of the vegetable kingdom, based upon this method of serum diagnosis. Such a " family tree " is shown in Fig. 112.

The phylogenetic relationships shown in this chart are in most instances similar to or identical with those which have been generally accepted by taxonomists. In some instances the serum-diagnostic method of Mez indicates relationships different from those usually accepted. The author, however, has discussed this chart with several taxonomic botanists and in each instance has received the assurance that the chart may well express the true phylogenetic relationships. It would accordingly seem as if serum diagnosis may be used in questions of disputed phylogeny.

[10] Practically all of these papers are published in *Botanischen Archivs* under the general title " Sero-diagnostische Untersuchungen." A summary of this work will appear in the Transactions of the International Congress of Plant Science held at Ithaca, New York, August, 1926.

[11] Mez, C., and Ziegenspeck, H., Zur Theorie der Sero-Diagnostik, *Bot. Arch.*, 12 : 163–202 (1925),

CHAPTER XX

NITROGEN BASES

A DISCUSSION of the nitrogen bases need not necessarily be considered as a part of protein chemistry. It seems probable, however, that either the nitrogen bases represent metabolic products formed by the breaking down of amino acids in the plant or animal organism, or that amino acids enter into the synthesis of these compounds. The nitrogen bases, therefore, may be looked upon as either amino acid decomposition products or products which have been derived from amino acids through some vital process.

It will be impossible in the space at our disposal to consider all of the nitrogen-containing compounds that have been isolated from plant or animal material. Only a few of the more important will be mentioned and only a few of the reactions involved will be considered.

THE DECOMPOSITION PRODUCTS OF AMINO ACIDS.—Proteins are hydrolyzed by fungi and bacteria to their constituent amino acids which are then acted upon by the micro-organism to yield either bases or acids. Deamination occurs both with anaerobes and aerobes, the characteristic *aerobic* reactions being either the production of a saturated acid possessing one less carbon atom than the original amino acid or the production of a hydroxy acid having the same number of carbon atoms as the original amino acid.

$$R—CH(NH_2)COOH + O_2 = R—COOH + CO_2 + NH_3$$
$$R—CH(NH_2)COOH + H_2O = R—CH(OH)COOH + NH_3$$

In some instances the above reaction is modified, producing instead an alcohol with the elimination of carbon dioxide and ammonia. Ehrlich[1, 2] has shown that this reaction occurs when amino acids are present in a medium where yeast is actively fermenting sugars, and has

[1] Ehrlich, F., Über die Vergärung des Tyrosins zu *p*-Oxyphenyl-äthylalkohol (Tyrosol), *Ber.*, 44 : 139–146 (1911).

[2] Ehrlich, F., and Pistschimuka, P., Überführung von Aminen in Alkohole durch Hefe- und Schimmelpilze, *Ber.*, 45 : 1006–1012 (1912).

used this reaction to prepare such rare alcohols as β-hydroxy-phenyl-ethyl alcohol (tyrosol), etc.,

$$\underset{\text{Tyrosine}}{OH(p)C_6H_4—CH_2—CH(NH_2)COOH} + H_2O = \underset{\text{Tyrosol}}{OH(p)C_6H_4—CH_2—CH_2OH} + NH_3 + CO_2$$

The characteristic *anaerobic* changes may be either (*a*) deamination and reduction or (*b*) decarboxylation. Deamination and reduction form a saturated acid containing the same number of carbon atoms, or deamination may take place without reduction and an internal rearrangement will cause the formation of an unsaturated acid (cf. Raistrick).[3]

$$R—CH(NH_2)COOH + H_2 = R—CH_2COOH + NH_3$$
$$R—CH_2—CH(NH_2)COOH \rightarrow R—CH = CH—COOH + NH_3$$

Decarboxylation results in the formation of amines and is a typical reaction of the putrefying bacteria,

$$R—CH(NH_2)COOH \rightarrow R—CH_2—NH_2 + CO_2;$$

or formic acid may be liberated, in which case reduction takes place with the formation of an amine having one less carbon atom than the original amino acid,

$$R—CH(NH_2)COOH + H_2 \rightarrow R—CH_2—NH_2 + H—COOH.$$

Some workers believe that decarboxylation always involves both of the above reactions, inasmuch as carbon dioxide and formic acid are usually formed simultaneously. The precise conditions for amine and acid formation under anaerobic conditions have not been accurately worked out. It seems probable that both of these reactions proceed simultaneously, the preponderance of one or the other being determined by the particular type of organism which is involved. In only a few instances have the products of the reaction been investigated for some specific organism.

The fundamental differences between aerobic and anaerobic putrefaction may be stated as *the elimination of nitrogen and the formation of acids by aerobic organisms and the elimination of carbon dioxide with the formation of bases by anaerobic organisms.* It is recognized that there are exceptions to this rule. By and large, however, the rule will hold.

[3] Raistrick, H., On a New Type of Chemical Change Produced by Bacteria. The Conversion of Histidine into Urocanic Acid by Bacteria of the Coli-Typhosus Group, *Biochem. J.*, 11 : 71–77 (1917).

Certain of the amines produced from amino acids by bacteria are of importance. Arginine yields urea and ornithine

$$(NH_2—CH_2—CH_2—CH_2—CH(NH_2)COOH),$$

ornithine in turn loses carbon dioxide to form *putrescine*

$$(NH_2—CH_2—CH_2—CH_2—CH_2—NH_2),$$

and lysine loses carbon dioxide to form *cadaverine*, the next higher homologue of putrescine. These bases have similar properties and are found together in the products of protein putrefaction. They were among the first compounds isolated from the putrefactive decomposition products of proteins and were originally classified as "ptomaines." The names are suggestive of undesirable compounds. The bases themselves are physiologically practically inert.

Tyrosine on putrefactive decomposition loses carbon dioxide to form the base, *tyramine*, β(*p*-hydroxyphenyl) ethylamine, the active principle of ergot. Histidine similarly forms *histamine* (β-imidazole ethylamine); tryptophane forms *tryptamine* (β-indole ethylamine); and arginine forms *agmatine* (δ-guanido butylamine). Tyramine, histamine, and tryptamine, all have a powerful physiological action. They are violent poisons. Tyramine is more or less similar to epinephrine, in that it raises the blood pressure, whereas histamine reduces arterial pressure to the shock level (30–40 cm. Hg) even when used in dosages as low as 1 mgr. per Ko. body weight. Histamine has been found by Abel to be the poison secreted by the skin of the African toad. Toad skin, according to Pliny, was a medicine. Abel showed that it contained a powerful drug. This is an additional instance where one of the old folk remedies was found to be not so foolish as it appeared to be.

Koessler and Hanke[4, 5] have published a long series of papers on the proteinogenous amines. The earlier laboratory method of preparation involved decarboxylation by *B-coli*.

Johnson and Daschavsky,[6] and Abderhalden and Gebelein[7] have shown that heating an amino acid with diphenylamine may be used as a

[4] Koessler, K. K., and Hanke, M. T., Studies on Proteinogenous Amines, I–V, *J. Am. Chem. Soc.*, 40 : 1716–1726 (1918); *J. Biol. Chem.*, 39 : 497–592 (1919).

[5] Koessler, K. K., The Relations of Proteinogenous Amines to Medicine, *Proc. Institute Med. of Chicago*, 3 : 46–62 (1920).

[6] Johnson, T. B., and Daschavsky, P. G., Researches on Amines. IX. The Formation of Tyramine by Decarboxylation of Tyrosine Produced from Silk, *J. Biol. Chem.*, 62 : 725–735 (1925).

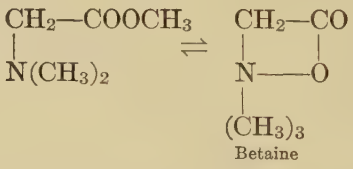
[7] Abderhalden, E., and Gebelein, F., Über Decarboxylierung von Aminosäuren unter Bildung der entsprechenden Amine und über die Darstellung der Enolform von 2,5-Dioxo-piperazinen, *Z. physiol. Chem.*, 152 : 125–131 (1926).

general method for the preparation of the corresponding proteinogenous amine. Using this method, Abderhalden was able to obtain 95 per cent of the theoretical amount of pure tyramine from tyrosine.

The odor of putrefying protein is due to indole and skatole (β-methyl indole) derived from tryptophane, and is not due to the bases noted above.

We have already noted that certain of these bases were classified originally as " ptomaines " and were supposed to be responsible for ptomaine poisoning. Most of the bases, however, are physiologically inert, and it is very doubtful if ptomaine poisoning is ever caused by any of these decomposition products of proteins. The common cause of ptomaine poisoning is the presence of the bacterial poison, *botulinus toxin*, secreted by *B. botulinus*. *Odor is no criterion of the presence or absence of botulinus toxin.* Completely rotted meat *may* be safe to eat, whereas apparently wholesome food may contain Botulinus toxin, if opportunity has been afforded for the organism to develop. Botulinus toxin is destroyed by heat, which probably accounts for the relatively few cases of ptomaine poisoning which actually occur.

The Betaines.—The betaines are a group of bases found in plants, which can be defined as *completely methylated amino acids.* The α, β, and γ amino acids form betaines. Those from α-amino acids are known as the α-betaines, those from β-amino acids, as β-betaines, etc. The simplest betaine is that of glycine, which is formed by an intramolecular rearrangement of the methyl ester of dimethyl amino acetic acid,



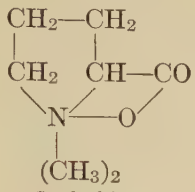
Betaine

forming an anhydride, the nitrogen atom being changed from the trivalent to the pentavalent condition. This betaine is physiologically inert. It occurs in the sugar beet and passes into the molasses in the process of sugar manufacture. Young sugar beets contain up to 2.5 per cent; old ones contain about 1 per cent of betaine. It likewise occurs in the leaves of many families of plants in quantities ranging from a fraction of 1 per cent to 3.78 per cent in *Atriplex canescens.*

Barger[8] has discussed the possible role of betaines in plants, suggesting that they are end products of nitrogen metabolism and that they

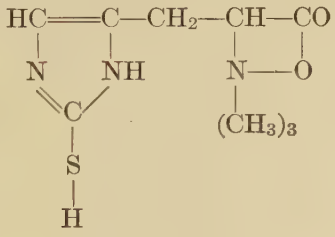
[8] Barger, G., The Simpler Natural Bases, Longmans, Green and Company, London (1914).

do not function in the vital processes. They are apparently most abundant in young or rapidly growing tissues.



Stachydrine

Stachydrine is the betaine of proline, and occurs in the leaves of the orange tree to about 0.19 per cent of the dry weight. Steenbock[9] found it to be present in the aqueous extract of alfalfa hay to the extent of about 0.5 per cent of the total nitrogen. Its chemical properties are such that in a Van Slyke analysis for nitrogen distribution it would be calculated in the *histidine* nitrogen fraction. Its physical properties are such, however, that using Kossel's method it would appear in the *lysine* fraction. These considerations emphasize the importance of recognizing the limitations of the various methods of protein analysis. *The methods of protein analysis are valid only when the material being analyzed is a pure protein.* In the event that non-protein nitrogenous compounds are present, neither the Van Slyke method nor any other method based upon a nitrogen determination will necessarily yield accurate values for any particular amino acid.



Ergothioneine
(Thiasine)

Benedict, Newton, and Behre[10] reported the isolation of a sulfur-containing nitrogenous base from blood. Newton, Benedict, and Dakin[11] have shown this to be identical with the base, *ergothioneine,*

[9] Steenbock, H., Isolation and Identification of Stachydrin from Alfalfa Hay, *J. Biol. Chem.*, 35 : 1–13 (1918).

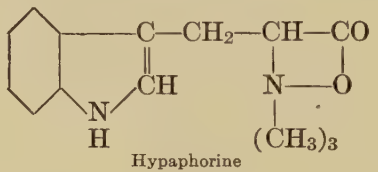
[10] Benedict, S. R., Newton, E. B., and Behre, J. A., A New Sulfur-Containing Compound (Thiasine) in the Blood, *J. Biol. Chem.*, 67 : 267–277 (1926).

[11] Newton, E. B., Benedict, S. R., and Dakin, H. D., The Chemical Constitution of Thiasine, *Science*, 64 : 602 (1926).

isolated by Tanret[12] from ergot and shown by Barger and Ewins[13] to be the betaine of thiolhistidine. Its function in the blood stream still remains to be determined.

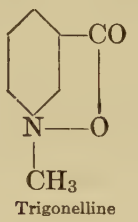
Trimethyl histidine, the betaine of histidine, has been isolated from certain edible mushrooms. Its chemical properties are such that it would appear in the lysine fraction of the Kossel separation.

Hypaphorine, the betaine of tryptophane, has been found in the seeds of *Erythrina hypaphorus*, a shade tree grown on the coffee plantations of Brazil. It has a very slight physiological action.



Hypaphorine

Trigonelline, the betaine of nicotinic acid is widely distributed in plants. It occurs in the garden pea. Physiologically it is inert.



Trigonelline

It seems very possible that a betaine corresponding to each of the known amino acids may well occur in nature. However, aside from their interest as naturally-occurring nitrogen-containing compounds, they appear to be of little biological importance.

Choline and Allied Substances.—Certain bases are formed by the bacterial decomposition of substances other than proteins. Among these are *choline* and its natural allies, *neurine* and *possibly muscarine*. These are all strong bases. β-amino ethyl alcohol is probably the precursor of choline which is trimethyl-β-hydroxy-ethyl-ammonium hydroxide, $(CH_3)_3$—N(OH)—CH_2—CH_2OH.

β-amino ethyl alcohol (NH_2—CH_2—CH_2OH) occurs as a constituent

[12] Tanret, Ch., Sur une base nouvelle retirée du seigle ergoté, l'ergothionéine, *J. Pharm. Chim.*, (VI) 30 : 145–153 (1909).

[13] Barger, G., and Ewins, A. J., The Constitution of Ergothioneine: a Betaine Related to Histidine, *J. Chem. Soc.*, London, 99 : 2336–2341 (1911).

of lecithin from various sources. While the mechanism by which this base is formed is still uncertain, there is a possibility that it may be derived by the loss of carbon dioxide from serine.

$$OH—CH_2—CH(NH_2)COOH \rightarrow OH—CH_2—CH_2—NH_2 + CO_2.$$

Lecithin (a phospholipide) is a *choline glycerol phosphoric acid ester*, and inasmuch as it occurs in all cells and especially in nervous tissues, it must be of marked physiological importance. Putrefaction of lecithin yields choline as one of the products. Choline has some physiological action; it is a depressant, causing a fall in blood pressure, and is antagonized by the alkaloid, atropine. Choline may occasionally occur in the free state in plants. Vinson[14] isolated a considerable quantity of choline from a fraction of the dilute sodium hydroxide extract of corn pollen. Whether or not the choline was present in the free state in the corn pollen or represented a decomposition product of the lecithin was not determined.

When putrefying bacteria act upon choline, the alcoholic side chain suffers dehydration, forming neurine, vinyl-trimethyl-ammonium hydroxide, $(CH_3)_3—N(OH)—CH=CH_2$. Neurine is exceedingly poisonous and may occur among the putrefaction decomposition products of meat and fish. Further decomposition results in the formation of trimethylamine.

According to the older literature, *muscarine* is the oxidation product of choline, where the alcohol group has been oxidized to an aldehyde. According to this view, muscarine would be trimethyl-ammonium hydroxide-acetaldehyde, $(CH_3)_3—N(OH)—CH_2—CHO$. King[15] questions this formula. He obtained 0.5 gram of muscarine aurichloride from 25.5 kilos of fresh *Amanita muscaria*. The gold content of this salt was 38.6 per cent, indicating a molecular weight of 210 for muscarine chloride. He states that the base is much more complex than is choline and that it should be classed with the more complex alkaloids. The base which he isolated was very toxic, 0.000002 gram being sufficient to kill a frog.

Muscarine is the poison of the "fly mushroom" or "fly agaric," *Amanita muscaria*. This mushroom was Caesar's favorite poison because it resembles so closely *Amanita caesarea* which is edible. He accordingly had *Amanita caesaria* served to himself, and *Amanita*

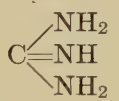
[14] Vinson, C. G., Some Nitrogenous Constituents of Corn Pollen, *J. Agr. Res.*, 35 : 261–278 (1927).

[15] King, H., The Isolation of Muscarine, the Potent Principle of Amanita Muscaria, *J. Chem. Soc.*, London, 121 : 1743–1753 (1922).

muscaria served on certain occasions to his guests. Muscarine poisoning is antagonized by atropine.[16]

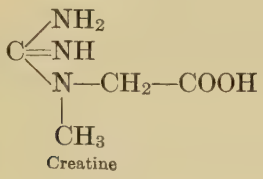
Miscellaneous Nitrogen Bases.—Only a few of the nitrogen bases occurring in plants or animals can be considered. For a more adequate discussion the reader is referred to the book by Barger, already noted.

Guanidine occurs occasionally in plants. It is found in the sugar beet and passes into the molasses in the process of sugar manufacture. It is probably originally derived from arginine.

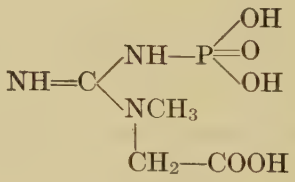


Guanidine

Creatine, methyl guanidine acetic acid, is a constituent of all vertebrate muscle. It occurs in the urine during starvation and is an indication that muscle tissue is being broken down. Recently Fiske and Subbarow[17] have isolated a creatine phosphoric acid complex from muscular tissue and point out that this compound is present in considerable proportion in resting muscle but is rapidly decomposed in fatigued or dead muscle. They suggest[18] a physiological role for the creatine and phosphoric acid in the muscle, *i.e.*, that they act as a buffer to bind the lactic acid formed from sugars by muscular activity.



Creatine



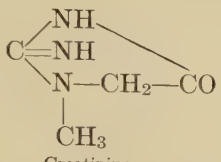
Phospho-creatine

[16] Warning should be inserted at this point, that while the poison of *Amanita muscaria* is antagonized by atropine, this is not the case for the "deadly Amanita," *Amanita phalloides*. *Amanita phalloides contains the Amanita toxin for which there is no antidote.* The toxin is a complex, possibly protein-like substance acting as a hemolysin, dissolving the red blood cells. It is one of the most poisonous of all plant products. One-half of an *Amanita phalloides* is sufficient for a fatal dose. As a rule, the symptoms of *Amanita* poisoning do not appear for some time after the mushroom has been eaten. For those who are interested in the properties of poisonous mushrooms, see Ford, W.W., and Clark, E. D., A Consideration of the Properties of Poisonous Fungi, *Mycologia*, 6 : 167–191 (1914) (60 references to literature).

[17] Fiske, C. H., and Subbarow, Y., The Nature of the "Inorganic Phosphate" in Voluntary Muscle, *Science*, 65 : 401–403 (1927).

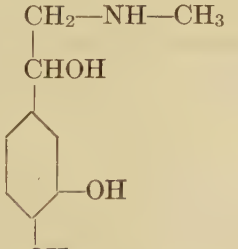
[18] Fiske, C. H., and Subbarow, Y., The Isolation and Function of Phosphocreatine, *Science*, 67 : 169–170 (1928).

Creatinine, the anhydride of creatine, occurs normally in all urine. The ratio of creatine to creatinine has been extensively studied as indicating relationships to physiological conditions of the individual. A discussion of such relationships, however, properly belongs in textbooks of physiological chemistry.



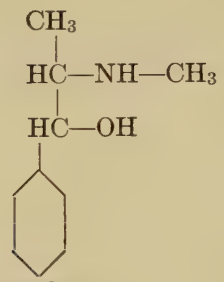
Creatinine

Another base of the utmost importance has three names which are rather common in the literature, *epinephrine* (Abel), *suprarenine* (Von Fürth), and *adrenaline* (Takamine). The consensus of opinion appears to be that this base was first isolated by Abel. On that consideration it should be known as epinephrine. Epinephrine is a derivative of pyrocatechol. It will be noted that the formula shows a marked resemblance to tyrosine. Undoubtedly epinephrine is one of the most important of all the naturally-occurring bases. It is the active principle of the suprarenal glands and regulates the blood pressure in the mammals. In health, about 0.1 per cent of the gland is epinephrine. The natural product is *l*-rotatory. The racemic mixture, containing the *d*- and *l*-modifications in equivalent amounts, has a much lower physiological activity than the natural product, the *d*-form having only approximately 5 per cent of the physiological activity of the *l*-form, again a striking example of biological specificity. An injection of as little as 0.0003 milligram per kilo of body weight is sufficient to produce a marked effect on blood pressure. The lethal dose (for man) is about 0.06 gram when injected intravenously. It contracts the blood vessels so that no blood can flow. Because of this marked contractile effect, epinephrine has come to be a valuable adjunct in surgery to control bleeding during a surgical operation.



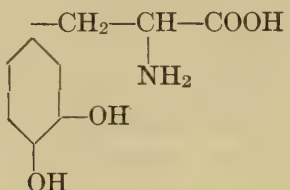
Epinephrine

Ephedrine, another base very similar in structure to epinephrine and having the formula[19] noted, has been isolated from the plant, *Ephedra equisetina*. This base has many of the desirable properties of epinephrine. It raises the blood pressure and has the advantage that it can be taken orally.



Ephedrine

3-4-Dihydroxy phenylalanine or "*dopa*" is an amino acid similar in some respects to epinephrine in structure. It occurs in the



Dopa

Georgia velvet bean (*Stizolobium deeringianum*) and in the seeds of *Vicia faba* (cf. Miller).[20] It reacts more or less like epinephrine but does not have the highly marked physiological properties, although it is distinctly toxic. It has not as yet been isolated from a protein. It is apparently the *chromogen* involved in certain brown and black animal pigmentations, such as in butterfly wings, *e.g.*, *Vanessa antiopa* (cf. Hasebroek[21]).

Carnosine, the dipeptide, β-alanyl histidine, has already been referred to. It is one of the principal products in " beef extracts," and may be

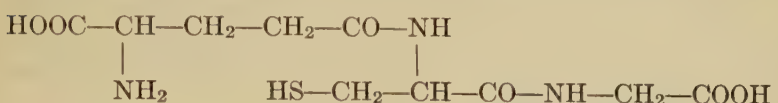
[19] Peterson, Joel B., Standardization of Ephedrine and Its Salts, *Ind. Eng. Chem.*, 20 : 388–391 (1928).

[20] Miller, E. R., Dihydroxyphenylalanine, a Constituent of the Velvet Bean, *J. Biol. Chem.*, 44 : 481–486 (1920).

[21] Hasebroek, K., Untersuchungen zum Problem des neuzeitlichen Melanismus der Schmetterlinge, I–X, *Fermentforschung*, 5 : 1–40, 297–333 (1921–22); 7 : 1–13, 139–142, 143–152, 183–194 (1923–24); 8 : 197–198, 199–226, 553–567, 568–573 (1925–26).

looked upon either as a dipeptide or as a base which is a constituent of muscle.

Glutathione, a tripeptide, glutamyl-cysteinyl-glycine, was isolated by Hopkins [22, 22a] from yeast, muscle, and mammalian liver. Such a tripeptide has twelve possible structural formulae. Kendall [22b] has shown that glutathione has the formula,



Glutathione (glutamyl-cysteinyl-glycine)

This appears to be the substance which is present in all cells that give the nitroprusside test. It is generally believed that glutathione acts as a hydrogen receptor and takes an active part in cell oxidations and reductions:

$$2\ R{-}SH \rightleftharpoons R{-}S{-}S{-}R + H_2$$

(cf. Dixon and Quastel,[23] Quastel, Stewart, and Tunnicliffe,[24] Tunnicliffe,[25] Stewart and Tunnicliffe [26]). Kendall and Nord [27] suggest that an unstable highly reactive oxygen addition product is formed between glutathione and oxygen, in which product the sulfur atom has a higher state of oxidation. This compound, together with the more stable —SH and —S—S—forms, make up a reversible oxidation-reduction system.

Spermine was isolated by Rosenheim [28] and has been shown by

[22] Hopkins, F. G., On an Autoxidisable Constituent of the Cell, *Biochem. J.*, 15 : 286–305 (1921).

[22a] Hopkins, F. G., On Glutathione: A Reinvestigation, *J. Biol. Chem.*, 84 : 269–320 (1929).

[22b] Kendall, E. C., McKenzie, B. F., and Mason, H. L., A Study of Glutathione. I. Its Preparation in Crystalline Form and Its Identification. *J. Biol. Chem.*, 84 :657–674 (1929). *Cf.* also "The Configuration of Glutathione," *Proc. Staff Meetings Mayo Clinic*, 4 : 359–360 (Dec. 11, 1929).

[23] Dixon, M., and Quastel, J. H., A New Type of Reduction-Oxidation System. Pt. I. Cysteine and Glutathione, *J. Chem. Soc.*, London, 123 : 2943–2953 (1923).

[24] Quastel, J. H., Stewart, C. P., and Tunnicliffe, H. E., On Glutathione. IV. Constitution, *Biochem. J.*, 17 : 586–592 (1923).

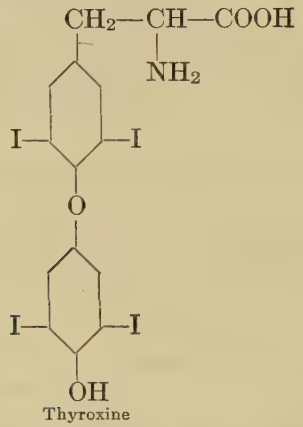
[25] Tunnicliffe, H. E., Glutathione. The Occurrence and Quantitative Estimation of Glutathione in Tissues, *Biochem. J.*, 19 : 194–198 (1925).

[26] Stewart, C. P., and Tunnicliffe, H. E., Glutathione. Synthesis, *Biochem. J.*, 19 : 207–217 (1925).

[27] Kendall, E. C., and Nord, F. F., Reversible Oxidation-Reduction Systems of Cysteine-Cystine and Reduced and Oxidized Glutathione, *J. Biol. Chem.*, 69 : 295–337 (1926).

[28] Rosenheim, O., The Isolation of Spermine Phosphate from Semen and Testis, *Biochem. J.*, 18 : 1253–1262 (1924).

Dudley, Rosenheim, and Starling[29] to be α-δ-bis (γ'-amino propyl amino)-butane, $NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$. It is apparently a constant constituent of sperm, although it has been found to occur in other animal organs. A similar compound, *spermidine*, was isolated by Dudley, Rosenheim, and Rosenheim[30] and has been shown by Dudley, Rosenheim, and Starling[31] to be α-(γ'-amino-propyl amino)-δ-amino butane, $NH_2(CH_2)_3NH(CH_2)_4NH_2$. It occurs in association with spermine in the various tissues and, as can be readily seen, is structurally related to spermine. The physiological function of these bases has not been determined.



Thyroxine

Thyroxine, the hormone of the thyroid gland, was isolated by Kendall[32, 33] as a decomposition product of the protein of the thyroid gland. Recently Harington,[34, 35] and Harington and Barger[36] have

[29] Dudley, H. W., Rosenheim, O., and Starling, W. W., The Chemical Constitution of Spermine, III. Structure and Synthesis, *Biochem. J.*, 20 : 1082–1094 (1926).

[30] Dudley, H. W., Rosenheim, M. C., and Rosenheim, O., The Chemical Constitution of Spermine. I. The Isolation of Spermine from Animal Tissues, and the Preparation of Its Salts, *Biochem. J.*, 18 : 1263–1272 (1924).

[31] Dudley, H. W., Rosenheim, O., and Starling, W. W., The Constitution and Synthesis of Spermidine, a Newly Discovered Base Isolated from Animal Tissues, *Biochem. J.*, 21 : 97–103 (1927).

[32] Kendall, E. C., Isolation of the Iodine Compound Which Occurs in the Thyroid, *J. Biol. Chem.*, 39 : 125–147 (1919).

[33] Kendall, E. C., Influence of the Thyroid Gland on Oxidation in the Animal Organism, *Ind. Eng. Chem.*, 17 : 525–534 (1925).

[34] Harington, C. R., Chemistry of Thyroxine. I. Isolation of Thyroxine from the Thyroid Gland, *Biochem. J.*, 20 : 293–299 (1926).

[35] Harington, C. R., Chemistry of Thyroxine. II. Constitution and Synthesis of Desiodo-Thyroxine, *Biochem. J.*, 20 : 300–313 (1926).

[36] Harington, C. R., and Barger, G., Chemistry of Thyroxine. III. Constitution and Synthesis of Thyroxine, *Biochem. J.*, 21 : 169–183 (1927).

synthesized thyroxine by known reactions and have shown that it has the formula which is indicated. Thyroxine has a very marked physiological action, exceptionally small amounts increasing the metabolism of the body. As little as 1 milligram of thyroxine injected intravenously may increase the metabolic rate as much as 2.5 per cent or as Kendall puts it, "The injection of 1 milligram of thyroxine will produce an increase in the carbon dioxide output of approximately 400,000 milligrams of carbon dioxide."

Following the injection of thyroxine, there is a pronounced delay in reaction, six to eight hours being required before the basal metabolism rate is affected. Following this time, there is a rapid increase in metabolic rate, but the maximal response may not be reached for several days following the injection. In a number of instances the maximal response to a single injection was not reached until the eighth or tenth day following the injection. The physiological effect of most drugs is over within a relatively short period of time. Thus, the effect of epinephrine may last, at the most, only a few hours. Kendall notes that a single injection of from 5 to 10 milligrams of thyroxine may affect the basal metabolism rate for a period as long as five to six weeks.

When certain pathological conditions influence the function of the thyroid gland so that thyroxine is no longer synthesized, as in the case of myxoedema and cretinism, the entire physiological process undergoes alteration which is particularly noted in the mental reactions, the individual either remaining essentially an idiot (cretinism) or losing his mental faculties (myxoedema). In each instance the mental faculties can be more or less completely restored and physiological processes brought back to normal by the use of thyroxine. Accordingly the isolation of this hormone in a form suitable for use in medicine must rank as one of the great contributions to modern medicine.

The Alkaloids.—Among the vegetable products, numerous oily or crystalline bases have been found, to which the term, "alkaloid," has been applied, and because of their physiological properties they have, for ages past, interested mankind.

When the orientals used opium or hashish, or the South American aborigines chewed the coca leaves for stimulation or the cinchona bark for fevers, they did not know that the reason they secured results lay in the alkaloidal content of the material. Only within the last few decades has the organic chemistry of certain of the alkaloids been elucidated. Due to the complex structure of the alkaloidal molecule, a study of the chemistry of the alkaloids is one of the most difficult fields of organic chemistry, and even today the structural formulae of such important drugs as strychnine and quinine are still more or less uncertain.

We can only consider briefly a few of the more important alkaloids. For those who are interested in the further development of this subject reference may be made to a number of books.[37-43]

It is difficult to formulate an exact definition of an alkaloid. The definition may be made so broad as to include all nitrogen-containing compounds or so narrow as to leave out compounds with definite alkaloidal properties. Ladenberg originally defined them as "*those naturally occurring vegetable substances of a basic character which contain at least one nitrogen atom forming a part of a heterocyclic ring.*" If we except the purine and pyrimidine bases, we can limit the definition to "basic substances found in plants and which contain a cyclic nitrogenous nucleus." Even this is too narrow, for a few compounds do not contain a "cyclic nitrogenous nucleus," nevertheless they may have the marked physiological properties of alkaloids. The modern chemist may well take exception to the words, "naturally-occurring." We have prepared synthetically medicinal substances which are *better* than the natural alkaloids, and these synthetic products are truly alkaloidal, both in chemical and physiological properties, if we accept chemical and physiological properties as criteria of alkaloids. No definition, accordingly, can be completely satisfactory. Ladenberg's definition is fairly satisfactory as applying to those alkaloids which occur in nature.

Historically, the work on alkaloids dates back to 1803 when Derosne isolated a crystalline compound from opium, which he called "opium salt." He did not, however, notice its basic character. In 1805, Sertürner, a German apothecary, isolated the material again independently, purified it, recognized its basic properties, and called it "morpheum." At the same time he separated an acid which he called "meconic" acid, and expressed the view that the two were combined in opium. These observations remained unnoticed until 1817, when Sertürner published a second paper, in which he further pointed out the basic character of morphine and described a number of its salts. Chem-

[37] Henry, T. A., The Plant Alkaloids, P. Blakiston's Son and Company, Philadelphia (1913).

[38] Pictet, A., The Vegetable Alkaloids, translated from the French by H. C. Biddle, John Wiley and Sons, New York (1904).

[39] Sidgwick, N. V., The Organic Chemistry of Nitrogen, Clarendon Press, Oxford (1910).

[40] Spiegel, L., Der Stickstoff, Fr. Vieweg und Sohn, Braunschweig (1903).

[41] Winterstein, E., and Trier, G., Die Alkaloide, Gebrüder Borntraeger, Berlin (1910).

[42] Schmidt, J., and Grafe, V., Alkaloide, Urban und Schwarzenberg, Berlin (1920).

[43] Blyth, A. W., Poisons: Their Effects and Detection, Third Edition, Charles Griffin and Company, Ltd., London (1895).

ists then began to look for other similar compounds, and in 1818, Pelletier and Caventou found strychnine in *Nux vomica*, followed by brucine in 1819, and in 1820 they isolated quinine and cinchonine from cinchona bark. At least two or three new alkaloids have been isolated and described each year since 1820.

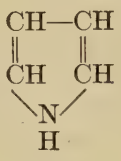
There was considerable speculation by the early chemists, as to the chemical constitution of these compounds, *e.g.*, Berzelius, Liebig, Hoffman, etc., but the first definite clue was obtained about 1842–46. Gerhardt, in 1842, distilled quinine, strychnine, and cinchonine with solid potassium hydroxide and obtained an oily base which he called "quinoleine"; later the name was changed to quinoline.

Previous to this, Runge (1834) had obtained a base from coal tar which he called " leucol," and Hoffman found that "quinoleine " and " leucol " were identical. Meanwhile, Andersen (1849–51) separated pyridine from bone oil, and later this base was isolated from the alkaloids, nicotine, coniine, piperidine, etc., by distillation with zinc dust. Isoquinoline was isolated from coal tar in 1885, and was later obtained from certain of the alkaloids, hydrastine, papaverine, etc. The fact that coal-tar bases could also be obtained from alkaloids stimulated research, and by breaking down alkaloids on the one hand, and building up derivatives of the coal-tar bases on the other, points of contact were established and information regarding the alkaloidal molecule was obtained. Eventually, some of the alkaloids were synthesized, and the recent advance in this field has been in the discovery of the *potent groupings* and then using these new groupings in new compounds which are better than the naturally-occurring alkaloids, in that *they have the same curative or medicinal properties and less of the undesirable properties.*

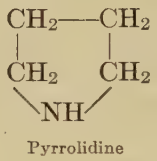
In using alkaloids in medicine, two properties of the alkaloid must be taken into consideration, *i.e.*, the minimal medicinal dose and the minimal lethal dose. The minimal medicinal dose is that dosage which will bring about the desired physiological effect. The minimal lethal dose is that dosage which may cause death. In a number of instances, alkaloids having very desirable physiological properties can not be used or can be used only with great caution, because of the fact that their physiological dose lies very close to their toxic dosage. Sometimes only three or four times the physiological dose is sufficient to cause death. The aim of the synthetic organic chemist interested in the field of the alkaloids is to ascertain those groupings which give to an alkaloidal molecule medicinal properties and those groupings which give to the alkaloid its toxic properties, with the hope of being able to synthesize new organic compounds having the desirable properties and lacking the

undesirable properties of the naturally-occurring alkaloids. The synthesis of *novocaine, procaine, butyn*, etc., have all been accomplished by studying the molecular configuration of cocaine and attempting to synthesize local anaesthetics having a similarity to certain parts of the cocaine molecule but lacking the toxic groupings.

A classification of the alkaloids has been made on the basis of the heterocyclic nucleus, although more than one heterocyclic ring may be present in the molecule of a single alkaloid. The following ring structures are the characteristic structures found in naturally-occurring alkaloids.

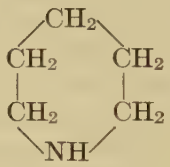


Pyrrole



Pyrrolidine

Pyridine



Piperidine

Quinoline

Isoquinoline

In most discussions of alkaloids, the *purine derivatives*, such as caffeine and theobromine, are considered as belonging to the alkaloid group. We have already noted these compounds as derivatives of the purine nucleus under nucleic acids and will not include them again at this point.

Before considering the organic chemistry of the alkaloids, it is perhaps justifiable to note certain of the families of plants in which alkaloids occur. Alkaloids are very unevenly distributed throughout the various groups of the plant kingdom. In some families of plants many alkaloids are found. Other families are noted by the practical absence of alkaloids. Very few of the monocotyledonous plants contain alkaloids, these compounds being confined almost exclusively to the dicotyledonous plants.

Among the angiosperms there are six families which are noteworthy for their alkaloidal content:

1. The *Apocynaceae* —" dogbane " (tropic and sub-tropic);
2. The *Leguminosae* —legumes;
3. The *Papaveraceae* —poppies;
4. The *Ranunculaceae*—buttercup and crowfoot;
5. The *Rubiaceae* —madder;
6. The *Solanaceae* —potato, tomato, nightshade.

Other families, such as the mints, roses, orchids, etc., sometimes, though rarely, contain alkaloids. They may occur in cell sap (opium), leaves (coca), stems, fruits (piperine, black pepper), seeds (*Nux vomica*), bark (*quinine*), roots (berberine in barberry roots), etc. Rarely does one alkaloid occur alone; usually two or three or more occur together; opium contains at least 20, and new ones are still being isolated from opium.

The alkaloids are usually solid and crystalline, but a few, like nicotine and coniine, are liquids. They are mostly colorless, although a few are yellow. They rarely occur free in the plant tissue, but as salts of organic acids, *e.g.*, malic, citric, succinic, oxalic, tannic, quinic, meconic, or aconitic, etc. In several groups of plants special alkaloids occur with special acids. Thus, the aconite alkaloids occur combined with aconitic acid, the opium alkaloids with meconic acid, and the cinchona alkaloids with quinic acid. Alkaloids readily form crystallizable salts with inorganic acids and are extracted from plant tissues by dilute sulfuric acid or hydrochloric acid. Certain of the alkaloids are volatile and may be steam-distilled from alkaline media; the non-volatile ones are set free by sodium hydroxide and extracted with ether, chloroform, etc., or may be adsorbed on Lloyd's reagent, as has already been noted in Chapter VI.

Various tests are used to detect the presence of alkaloids. Among the more common are, iodine in potassium iodide forming a yellow-brown precipitate (Mayer's reagent), platinic chloride forming a crystalline double salt which can be analyzed for platinum content, auric chloride forming a crystalline aurichloride (a similar crystalline double salt), and lead acetate yielding a precipitate.

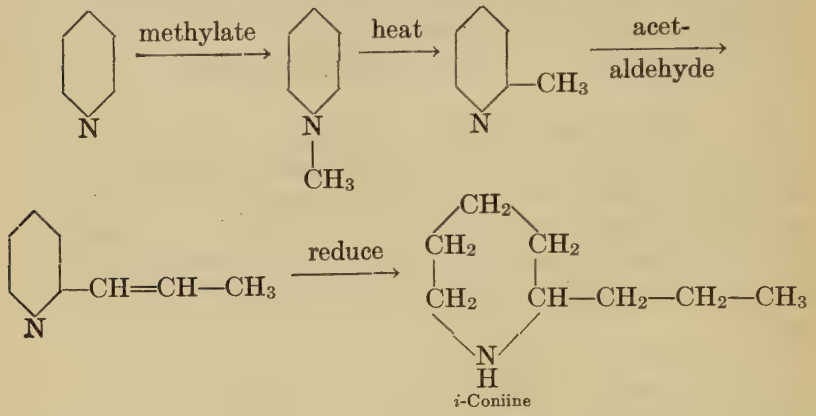
Most alkaloids are bitter, but this is not a necessary property, for piperine, from black pepper, is tasteless. (The pungent taste of pepper is *not* due to its alkaloidal content but to an essential oil.)

Many of the alkaloids contain an asymmetric carbon atom and are accordingly optically active. Most of them are *l-rotatory*. *d*-Tartaric

acid is usually used to resolve racemic mixtures, using the same technic as already noted for resolving amino-acid mixtures.

The Pyridine Group.—This group includes nicotine (tobacco), coniine (hemlock), piperine (black pepper), etc.

Coniine is α-*n*-propyl piperidine. This was the first alkaloid to be synthesized, Ladenberg, in 1886, accomplishing the synthesis.

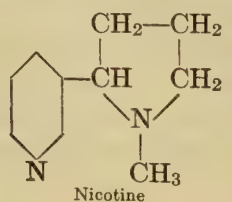


i-Coniine

Pyridine was methylated on the nitrogen, forming *n*-methyl pyridine, which when heated caused a migration of the methyl group to the α position. The α-picoline so formed condensed with acetaldehyde to form α-allyl pyridine, which on reduction yielded inactive coniine. The racemic mixture was separated by crystallization with *d*-tartaric acid. It would appear as if the synthesis could have been easily accomplished by adding a normal propyl group at the stage where the $—CH_3$ group was added to the pyridine. Ladenberg attempted to do this and found that when the aryl radical shifted from the nitrogen to the α-carbon, it became an isopropyl group, thus resulting in the synthesis of an isomer of coniine.

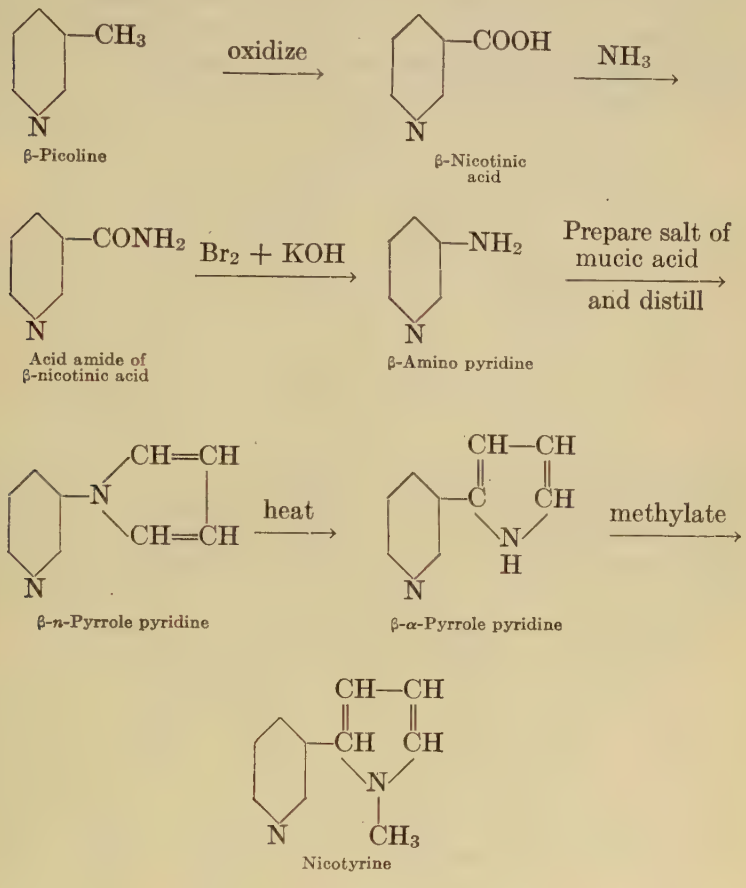
From the historical standpoint coniine is an interesting alkaloid, inasmuch as Socrates was supposed to have died from the effect of coniine in his drink of the deadly hemlock.

Nicotine, the alkaloid of tobacco, is β-(*n*-methyl pyrrolidine) pyridine. This alkaloid was synthesized by Amé Pictet during the period, 1895–



Nicotine

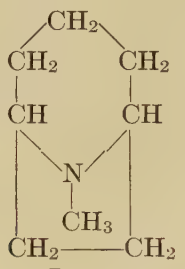
1904, starting from β-picoline (β-methyl pyridine) which is formed when glycerol, an organic nitrogen compound, and P_2O_5 are dry distilled. The mechanism of the reaction is unknown. The various steps in Pictet's synthesis are as follows:



Nicotyrine treated with iodine forms the di-iodo compound which reduces with zinc and potassium hydroxide to the di-hydro compound. The di-hydro compound with bromine gives the di-brom inactive nicotine, and the bromine can be removed from this by reducing with tin and hydrochloric acid, forming *inactive nicotine* which can be separated into its optical isomers. The trick in this synthesis was the reduction of the nicotyrine, reducing the pyrrole ring without reducing the pyridine ring. Solving the problem of reduction required nearly ten years of research.

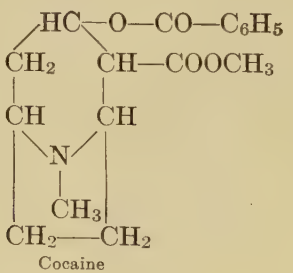
The Pyrrolidine Group.—The pyrrolidine group includes relatively few alkaloids. Only one of these is of any interest to us, *i.e.*, *stachydrine* which has already been mentioned as the betaine of proline.

The Tropane Group.—The tropane group contains a ring composed of the 6-carbon piperidine ring and the 5-membered pyrrolidine ring, and can be regarded as derivatives of the heterocycle, *tropane.* These alkaloids are found in the *Solanaceae* and in coca, and are represented by *atropine, hyoscine, hyoscyamine, cocaine,* etc.



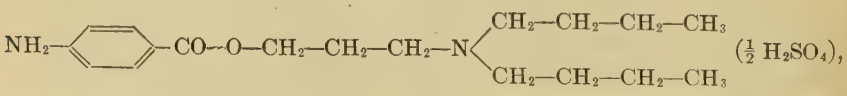
Tropane

Cocaine has been synthesized by Willstätter. Attempts to synthesize compounds having the desirable properties have yielded *novocaine,* $(C_2H_5)_2N—CH_2—CH_2—O—CO—C_6H_4—NH_2$ (*p*), which is only one seventh as toxic as cocaine. The local anaesthetic properties of cocaine



Cocaine

lie in the benzoic acid ester and nitrogen portion. Undesirable properties accompany the methyl ester group. Novocaine cannot be used as a surface anaesthetic. *Butyn,*

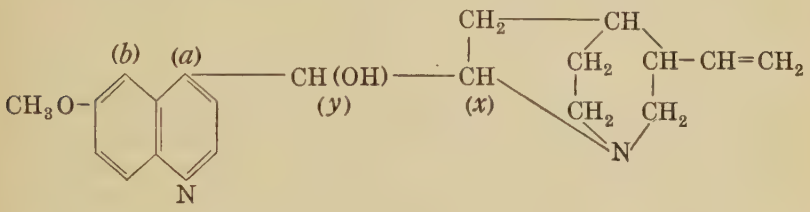


however, is fairly efficient in this regard.

For injection into the body it is desirable to have a water-soluble, neutral material, therefore, in the synthetic products the p-NH_2 group is added to the benzene nucleus. The hydrochloride of the synthetic compounds containing the p-amino group is essentially neutral.

The Quinoline Group.—This includes the alkaloids, *quinine, cinchonine, strychnine, brucine,* etc. None of these alkaloids have been synthesized, so their structure is not certainly known.

Quinine is probably,



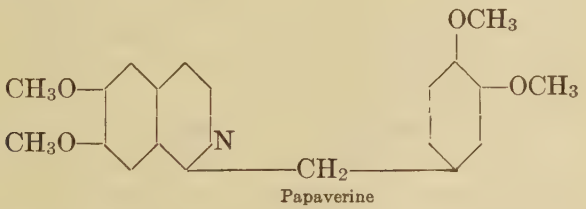
The OH at (y) may be at (x).

The union at (a) may be at (b).

If —OCH_3 is on the quinoline nucleus, the compound is *quinine*; if —OH is on the quinoline nucleus, the compound is *cuprine*, while if —H is on the quinoline nucleus, the compound is *cinchonine*. *Ethyl hydrocuprine* has the —OH on the quinoline nucleus and the —$CH{=}CH_2$ group is hydrogenated to —CH_2—CH_3. It has very desirable properties as a specific for *pneumococcus meningitis*, but the lethal dose is dangerously close to the curative dose, so that it is used only in case of emergency.

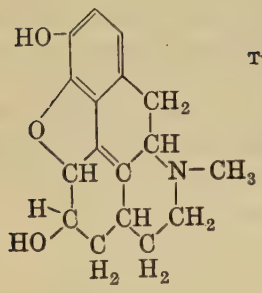
The exact formulae for *strychnine* and *brucine* are more imperfectly known than is that for quinine. Brucine is di-methoxy strychnine.

The Isoquinoline Group.—The isoquinoline alkaloids occur mainly in the opium series.

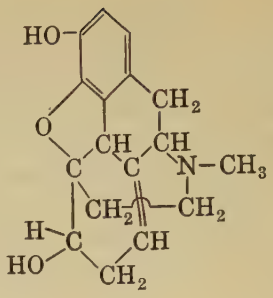


Papaverine

Papaverine is one of the simplest of these; *morphine* is probably one of the most complex.



Two suggested alternative formulae for morphine ($C_{17}H_{19}NO_3$)

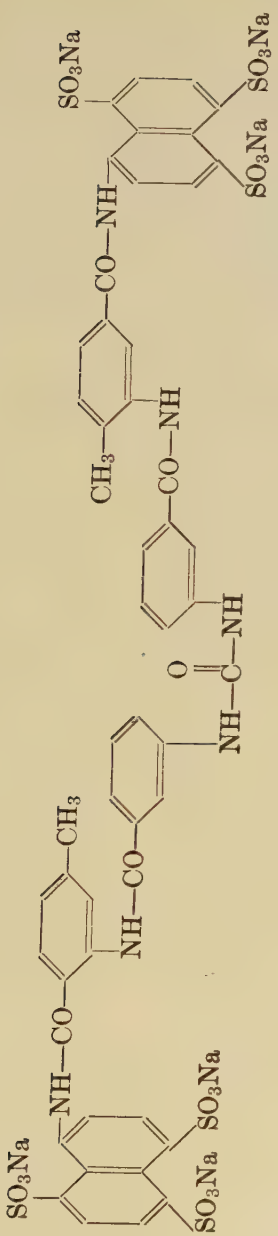


The recent announcement of a specific for the African sleeping sickness is the result of organic chemical research, not based upon the study of any particular alkaloid. The question accordingly arises as to whether or not mention of such a compound ought to be made in a section devoted to alkaloids. It seems to the author, however, that a synthetic product possessing more or less of an alkaloidal type of structure might well be considered, particularly in view of its immense importance in combating the ravages of the tsetse fly in certain regions of Africa. It is estimated that 500,000 dollars was spent in experimental research by the Baeyer Company of Germany before " Baeyer 205 " was perfected. " Baeyer 205 " appears to be identical in formula with the French " Fourneau 309," and to have the formula on page 465.

The therapeutic dose is only 1/160 of the lethal dose. It is, therefore, reasonably safe to administer. It is water-soluble, apparently cures the " sleeping sickness," and is stated to confer an immunity which lasts for months.

So much for structural formulae. Only a very few of the alkaloids have been sufficiently investigated to be sure as to what their decomposition products are and to make even a guess at their structural formulae. Naturally, those alkaloids which are important in medicine have attracted the most attention, but the elucidation of such complicated formulae is one of the most difficult tasks in organic chemistry. The expense connected with securing an adequate amount of material to work with is also an important item.

Another compound, which may be considered as intermediate between the true alkaloids and other compounds containing a basic nitrogen group, is *solanine*. Solanine occurs in the potato, and on acid hydrolysis it yields one molecule each, of dextrose, galactose, and rhamnose, and a base, *solanidine* ($C_{40}H_{61}NO_2$), concerning which very little is known except that it contains two —OH groups. Solanine is,


SO3Na
SO3Na
SO3Na
SO3Na
NH—CO
CH3
NH—CO
CH3
CO—NH
CO—NH
O
NH—C—NH

therefore, possibly an alkaloidal glucoside. Solanine is obtained commercially from the juice of potato sprouts. Under ordinary conditions, potatoes seldom ever contain enough to produce toxic effects, but certain strains of potatoes contain appreciable amounts. " Sunburned " potatoes are nearly always bitter and contain an increased quantity of solanine. Death may result, if a considerable quantity of such tubers are eaten, especially if they are baked and eaten "in their jackets." Potatoes normally contain about 0.024 parts of solanine per 1000, but sunburned potatoes have been found to contain as high as 0.588 parts per 1000. Approximately 70 per cent of the solanine is removed in the parings. (Cf. Bömer and Mattis,[44] Rothe, [45] and Behre and Ehrecke[46] for references to cases of poisoning by solanine and for methods for detecting and estimating solanine.)

The Origin of Alkaloids in Plants.—Obviously the question of origin and purpose of such compounds as the alkaloids, in the economy of a plant, is a question which will probably always remain a subject of speculation. Inasmuch as they do not occur in all forms of plant life, it is obvious that they are not essential to the life process of a plant. The great variability in amount from season to season and from locality to locality also indicates that they may possibly be regarded as a by-product of the synthetic and metabolic activities of the plant. This is the view of Pictet, who believes that the nitrogen residues from protein utilized by the plant in its metabolic processes are resynthesized into alkaloids (by certain plants which possess such synthetic power), because in this form the alkaloids are less harmful to the plant than are the direct decomposition products of the proteins. Plants do not excrete their waste products, so do not synthesize any considerable quantities of urea or uric acid. The above view is obviously only a guess. We have no evidence as to the real significance of the alkaloids in the organs or tissues of a plant. It has been suggested that they are a form of protection, so that animals will not eat them, or if they do eat them, they will be " punished " and die. Such a view, however, attributes rather too much intelligence to a plant!

The alkaloidal content may be increased or decreased by appropriate selection of strains, by plant breeding, and by fertilizing (especially nitrogen and phosphorus). Collecting at the proper time is also an

[44] Bömer, A., and Mattis, H., Über höhe Solaningehalte bei Kartoffeln, *Z. Nahr. Genussm.*, 45 : 288–291 (1923); Der Solaningehalt der Kartoffeln, *Z. Nahr. Genussm.*, 47 : 97–127 (1924).

[45] Rothe, J. C., Über Erkrankungen nach Genuss von Solaninhaltigen Kartoffeln, *Z. Hyg. Infektionskrankh.*, 88 : 1–12 (1919).

[46] Behre, A., and Ehrecke, H., Solaningehalt von Kartoffeln, *Chem. Ztg.* 42 : 593 (1918).

important factor. Thus, the first lancings of the poppy capsules yield an opium containing a very much greater morphine content than do the later lancings. (*Cf.* Annett.[47-49])

The nicotine content in tobacco has been increased by plant breeding and manuring. This is desirable in certain instances in order to secure a high nicotine content for spray manufacture.

[47] Annett, H. E., Factors Influencing Alkaloidal Content and Yield of Latex in the Opium Poppy (*Papaver somniferum*), *Biochem. J.*, 14 : 618–636 (1920).

[48] Annett, H. E., Investigations on Indian Opium, No. 2. The Effect of Environmental Factors on the Alkaloidal Content and Yield of Latex from the Opium Poppy (*Papaver somniferum*) and the Bearing of the Work on the Functions of Alkaloids in Plant Life, *Mem. Dept. Agr. India, Chem. Series*, 6 : 61–154 (1921).

[49] Annett, H. E., and Singh, H. D., Investigations on Indian Opium, No. 4. Further Experiments on the Influence of Manures on the Yield and Morphine Content of the Latex from the Opium Poppy, *Mem. Dept. Agr. India, Chem. Series*, 8 : 27–37 (1925).

III

CARBOHYDRATES

"Für das Studium der chemischen Prozesse im Tier- und Pflanzenkörper ist nächst den Eiweisskörpern keine Gruppe von Kohlenstoffverbindungen so wichtig, wie die Kohlenhydrate, und als Nahrungsmittel nehmen sie unstreitig die erste Stelle ein."

EMIL FISCHER (1890)

CHAPTER XXI

GENERAL CONSIDERATIONS—THE SYNTHESIS OF CARBOHYDRATES IN NATURE AND THE CLASSIFICATION OF CARBOHYDRATES

Optical Rotation.—Light may be defined for our purposes as "radiant energy, propagated in free space with the velocity common to all radiant energy but having wave lengths such that it affects the human eye." It is obvious that the vibrations comprising light may take place in all directions in a plane perpendicular to the path of the beam of light. However, certain minerals, such as tourmaline, transmit light through the crystal but allow it to pass only in a definite plane, *i.e.*, perpendicular to the laminae of the crystal, so that light vibrating in only a single plane can pass through the crystal. The light which does pass is *plane polarized light*, although it is commonly referred to simply as *polarized light.*

The action of a polarimeter may be illustrated by using two books and a ruler. If the two books are lying end to end on a table, a ruler may pass between the leaves of both and project at each end. Here, there is no resistance to the ray of light passing through the two crystals. If, however, one of the books is rotated so that the planes of the leaves do not coincide with the planes of the leaves of the other book, the ruler can no longer be pased through both volumes without being bent or twisted and the ruler must be twisted through the same angle as that through which the book was rotated.

Biot, in 1815, discovered that certain substances, such as turpentine (liquid) or a solution of sugars or tartaric acid, *rotated* the plane of polarized light. The angle of rotation is determined by use of the polarimeter which contains two prisms of tourmaline in a form known as "Nicol prisms." These prisms are set in such a manner that light readily passes through both prisms from the light source to the eye of the observer. Then a cylinder containing the liquid under investigation is placed between the two Nicol prisms, and if the solution rotates the plane of polarized light, the field becomes black. The second Nicol prism is now rotated until light again passes through, and the number of degrees through which the prism was rotated is read from the scale.

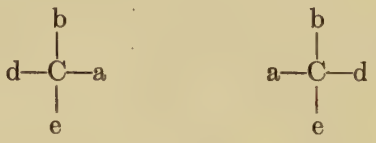
In making studies of optical rotation it is very desirable to use monochromatic light, *i.e.*, light of a single wave length. Ordinarily the sodium light is used, inasmuch as sodium light is largely that of the D sodium line in the yellow part of the spectrum. The mercury vapor lamp is, however, coming into rather general use. Here we are dealing with several lines close together in the green portion of the spectrum.

The optical rotation of a substance is usually expressed as the *specific rotation* which is defined as *the number of degrees of angular rotation produced in a one decimeter tube containing a solution of one gram of substance in* 1 *cc. volume.*

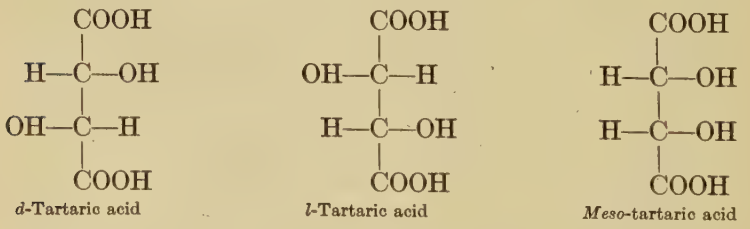
Optical Isomerism.—Certain substances possess the physical properties of optical activity when they are (*a*) in the crystalline state, *e.g.*, quartz, (*b*) in solution, *e.g.*, sugars, or (*c*) in the liquid form, *e.g.*, lactic acid, turpentine, etc.

Pasteur discovered that optical activity occurred in more than one form, *e.g.*, that tartaric acid could exist in both *dextro* and *levo* modifications and that the one rotated the plane of polarized light to the right and the other rotated it to the left. He also showed that racemic acid was an equal mixture of the *d* and *l* forms.

Van't Hoff (1874), and Le Bel (1874) independently advanced the theory of an asymmetric carbon atom where four different groups are attached to the same carbon atom.

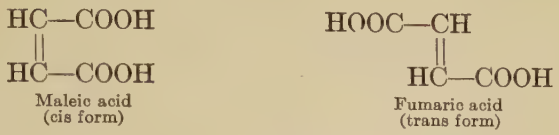


Here we have two substances which are mirror images of each other and yet are structurally different. Thus, for tartaric acid we have the three possibilities,



and additional evidence for these structures is that we can secure tartaric acid from maleic and fumaric acids by oxidation with potassium per-

manganate. Maleic and fumaric acids differ only in their space configurations.



Maleic acid (cis form) Fumaric acid (trans form)

Maleic acid yields only *meso*-tartaric acid. Fumaric acid yields a racemic mixture of *d*- and *l*-tartaric acids. *Meso*-tartaric acid is *internally compensated*, having in the formula a top carbon the same as in the *d* form and a bottom carbon of the *l* form. Consequently one carbon is *d* and the other is *l*, and the rotations *within the molecule* neutralize each other, so that no rotation of the light results. Naturally-occurring tartaric acid is the *d* form, and its purity is usually determined by the optical rotation of a solution of the acid. In certain samples of synthetic tartaric acid (from starch) there has been found to be present a small amount of *meso*-tartaric acid, and the lower optical rotation of this mixture indicated an impure acid, although titration with standard alkali indicated that it was pure. The presence of the *meso* acid caused, in this instance, a great deal of annoyance.

When, as in the carbohydrates, the chemical groupings at the opposite ends of the carbon chain are different, the number of possible isomers and of racemic mixtures due to asymmetric carbon atoms can be calculated from the formulae:

$$N = 2^n \tag{125}$$

and

$$r = \frac{N}{2} \tag{126}$$

where N = number of isomers;
n = number of asymmetric carbon atoms;
r = number of racemic mixtures.

The following table shows that as the number of asymmetric carbon atoms in such compounds increases, there is a very great increase in the number of possible isomeric forms.

n =	1	2	3	4	5	6	7	8
N =	2	4	8	16	32	64	128	256
r =	1	2	4	8	16	32	64	128

In the more recent formulae for the carbohydrates we have *five* asym-

metric carbon atoms in a C_6 sugar. Consequently 32 C_6 sugars are possible, *all of which will have exactly the same chemical composition.*

Carbohydrates are remarkable because of their optical activity. Using the free —CHO formula, there are 16 possible isomeric aldohexoses, $C_6H_{12}O_6$. Fourteen have been synthesized. *Only 3 occur in nature, d-glucose, d-mannose,* and *d-galactose.* This natural limitation of the number of hexoses produced by the plant or utilized by either the plant or animal kingdom is extremely interesting, as indicating a selective process during the period of organic evolution. It also indicates a rather remarkable unanimity in the evolutionary process as applied to nature as a whole.

It should be noted at this point that the prefixes, *d* and *l* (*e.g.*, *d*-mannose, $[\alpha]_D$ $-14.6°$, *d*-fructose $[\alpha]_D^{20}$ $-93.5°$, and *d*-glucose $[\alpha]_D^{20}$ $+52.5°$), have entirely different meanings *insofar as sugar chemistry is concerned* from their meanings in other fields of chemistry. In all other branches of chemistry they refer to the direction of the rotation of the plane of polarized light, *i.e.*, whether the rotation is clockwise or counter-clockwise. This is not necessarily true for the carbohydrates. *A d-sugar is a sugar which is structurally related, insofar as the asymmetry of its carbon atoms are concerned, to d-glucose. An l-sugar is similarly structurally related to l-glucose.* Accordingly a number of the known sugars may belong to the *d* series and still may rotate the plane of polarized light counter-clockwise. Similarly certain of the *l* sugars may have a pronounced dextro rotation. Therefore, *d* and *l* as prefixes to the names of the carbohydrates signify only their family relationships and bear no necessary reference to their optical rotation, excepting in the case of *d*- and *l*-glucose, to which all of the others are referred.

This nomenclature was introduced by Emil Fischer during his studies of the synthesis of carbohydrates. It is without doubt a confusing element in carbohydrate study. Nevertheless, this particular nomenclature, insofar as the carbohydrates are concerned, is so firmly fixed in the literature that it seems impracticable to alter it. It is to be regretted that Fischer did not use some other symbols to designate structural relationships.

The Importance of Carbohydrates.—Carbohydrates and proteins play the major role in both plant and animal life. *The carbohydrates are the fuel which serves to carry on the vital processes.*

Interest in carbohydrates naturally centers around glucose, for this is one of the most important of the sugars, probably the most important. Whether or not it is the primary product of carbohydrate synthesis in the plant is still uncertain. It is nevertheless the unit from which are derived, sucrose, maltose, starch, glycogen, and cellulose. The bulk of

carbohydrates in our body metabolism is transported as glucose, and in the form of glucose the plant transports its carbohydrates.

The carbohydrates as a group constitute three-fourths of the dry weight of the plant world. Something like 30 sugars have been isolated from plant or animal sources.

The Synthesis of Carbohydrates in Nature.—The mode of synthesis of carbohydrates in the plant is still uncertain. We know that in the presence of sunlight and chlorophyll, under the conditions which are present in green leaves, carbon dioxide and water unite to form carbohydrates, and that oxygen is evolved during the synthesis. This particular chemical reaction is without doubt the most important chemical reaction taking place on the earth, for by this reaction both plants and animals have made available to them a source of energy.

One cannot emphasize too strongly that in the plant the synthesis of all organic substances must revert to the initial mechanism involved in the synthesis of carbohydrates, so that the synthesis of proteins, fats, alkaloids, acids, etc., is in the last analysis dependent upon the photosynthetic mechanism.

Numerous theories have been proposed to explain the mechanism which takes place. These theories have been adequately discussed by Spoehr[1, 2] (cf. also Meldola),[3] so that we will only mention in a casual way two of the more important of the theories.

The Formic Acid Theory.—This theory assumes that formic acid is the initial product of photosynthesis, the carbon dioxide undergoing reduction to formic acid with the formation of hydrogen peroxide, the formic acid being then transposed into carbohydrates,

$$CO_2 + 2H_2O = H—COOH + H_2O_2.$$

Although this theory has received some support in recent years, it must still remain a theory without experimental proof.

The Formaldehyde Theory.—The formaldehyde theory is perhaps the oldest of the theories concerning the mechanism of the process, appears the most plausible, and has attracted the attention of the most investigators.

Butlerow, in 1861, observed that trioxymethylene in the presence of

[1] Spoehr, H. A., Photosynthesis, Chemical Catalog Company, Inc., New York (1926).

[2] Spoehr, H. A., The Theories of Photosynthesis in the Light of Some New Facts, *Plant World*, 19 : 1–16 (1916).

[3] Meldola, R., The Living Organism as a Chemical Agency; A Review of Some of the Problems of Photosynthesis by Growing Plants, *J. Chem. Soc.*, London, 89 : 749–770 (1906).

alkali was polymerized to a sugar (formose), $6HCHO \longrightarrow C_6H_{12}O_6$. From this sugar Fischer (*vide infra*) later synthesized the naturally-occurring sugars.

Baeyer,[4] in 1864, put forth the hypothesis that formaldehyde in solution was in reality methylene glycol, $CH_2(OH)_2$, and that Butlerow's reaction should be written, $6CH_2(OH)_2 = C_6H_{12}O_6 + 6H_2O$. Baeyer believed that formaldehyde was the initial product in photosynthesis, that the carbon dioxide was first reduced to carbon monoxide and oxygen ($CO_2 = CO + O$), and that carbon monoxide was then reduced to formaldehyde ($CO + H_2 = HCHO$), which then condensed to a sugar, possibly through the intermediate stages of glycolic aldehyde, $CH_2OH—CHO$, or of glyceric aldehyde, $CH_2OH—CHOH—CHO$. This hypothesis has interested investigators along three different lines, (1) the reduction of carbon dioxide in aqueous solution to formaldehyde by chemical and photochemical means, (2) a search for formaldehyde or other intermediate products in green plants during active photosynthesis, and (3) formaldehyde as a source of energy for plant life.

Researches along all three of these lines have yielded valuable results, the only question at issue being the interpretation of *in vitro* experiments to *in vivo* action.

Fenton,[5] in 1907, showed that carbon dioxide could be reduced by appropriate means to formaldehyde. Within recent years the use of catalysts has confirmed this observation and bids fair to build a chemical industry upon this reduction process. However, the conditions existing in the plant are so different from the conditions used for the industrial reduction of carbon dioxide, that the question arises as to whether the plant possesses the mechanism for the production of formaldehyde in this manner.

Probably no group of papers has interested the plant physiologist, the biochemist, the photochemist, and the physical chemist, as has the recent series of papers by E. C. C. Baly[6-9] and his coworkers. These papers Baly has summarized in a more or less general paper.[10]

[4] Baeyer, A., Ueber die Wasserentziehung und ihre Bedeutung für das Pflanzenleben und die Gährung, *Ber.*, 3 : 63–75 (1870).

[5] Fenton, H. J. H., The Reduction of Carbon Dioxide to Formaldehyde in Aqueous Solution, *J. Chem. Soc.*, London, 91 : 687–693 (1907).

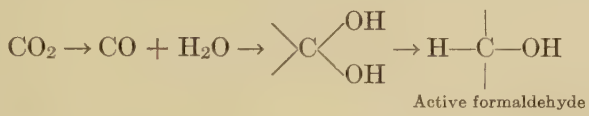
[6] Baly, E. C. C., Heilbron, I. M., and Barker, W. F., Photocatalysis. Pt. I. The Synthesis of Formaldehyde and Carbohydrates from Carbon Dioxide and Water, *J. Chem. Soc.*, London, 119 : 1025–1035 (1921).

[7] Baly, E. C. C., Heilbron, I. M., and Hudson, D. P., Photocatalysis. Pt. II. The Photosynthesis of Nitrogen Compounds from Nitrates and Carbon Dioxide, *J. Chem. Soc.*, London, 121 : 1078–1088 (1922).

[8] Baly, E. C. C., Heilbron, I. M., and Stern, H. J., Photocatalysis. Pt. III.

Baly believes that formaldehyde is the primary product of photosynthesis. Using quartz vessels and the light from a quartz mercury vapor lamp, he states that he was able to synthesize formaldehyde from carbon dioxide and water, when the system was exposed to the short wave lengths of light. He found that he could polymerize this formaldehyde to a sugar by the action of the longer wave lengths of light. He accordingly suggests that the function of the chlorophyll is to catalyze the reaction or to act as a transformer of energy so that the longer wave lengths of light can effect the synthesis of formaldehyde. Sugar synthesis, according to Baly, is a two-phase action, the formation of the so-called active formaldehyde, followed by the polymerization of this product into carbohydrates. We have already noted (p. 307) Baly's suggested scheme for the natural synthesis not only of carbohydrates but of amino acids, proteins, alkaloids, etc., and that his scheme is supported by his *in vitro* experiments.

In his scheme, carbon dioxide is reduced to carbon monoxide which unites with water to form " active " formaldehyde,



It will be noted that the carbon in active formaldehyde is divalent. Accordingly it is very reactive, undergoing polymerization or readily uniting with other reactive groups.

Baly's experiments have been criticized by Porter and Ramsperger[11] who failed to secure any evidence for the formation of formaldehyde or of sugars by radiating pure solutions of carbon dioxide in conductivity water in quartz with ultraviolet light. They suggest that Baly's results may have been due to traces of organic substances derived from stopcock grease, rubber tubing, etc. It must be admitted, however, that positive results in this field of research are somewhat more significant than purely negative results.

In later work, Baly considers the arguments presented by Porter and Ramsperger, and appears to successfully refute them. He notes, how-

The Photosynthesis of Naturally Occurring Nitrogen Compounds from Carbon Dioxide and Ammonia, *J. Chem. Soc.*, London, 123 : 185–197 (1923).

[9] Baly, E. C. C., Photosynthesis and the Functions of Pigments in the Living Plants, *J. Soc. Dyers and Colourists*, 38 : 4–9 (1922).

[10] Baly, E. C. C., Photosynthesis, *Ind. Eng. Chem.*, 16 : 1016–1018 (1924).

[11] Porter, C. W., and Ramsperger, H. C., The Action of Ultraviolet Light upon Carbon Dioxide and Water, *J. Am. Chem. Soc.*, 47 : 79–82 (1925).

ever, that formaldehyde is only an evanescent product, present during the irradiation but being so active that it decomposes following the cessation of the irradiation.

Recently, Baly[12-14] has extended his original observations and announces the synthesis of carbohydrates from carbonic acid by means of visible light, thus approximating natural conditions. He has summarized these recent experiments in a popular article,[15] pointing out that the presence of finely divided materials in the solution not only promoted the rate of synthesis in ultraviolet light but that in the presence of certain of these suspended materials formaldehyde and carbohydrates were formed in the visible portion of the spectrum. Baly suggests that the synthetic reaction may have occurred on the *surface* of the quartz vessel and that surface area may have been the determining factor. He accordingly suspended pure aluminum powder in water in a quartz vessel and maintained it in suspension by a stream of carbon dioxide, the whole being exposed to ultraviolet radiation. He announces an increased yield of carbohydrates, which in his experiment was approximately equal to the weight of aluminum powder which was used. Identical results were obtained with other powders, such as aluminum hydroxide and the carbonates of aluminum, zinc, and magnesium. Nickel carbonate under the same conditions gave a larger yield of carbohydrates, when the system was illuminated with the light from an ordinary tungsten filament lamp, than did any of the white powders illuminated with ultraviolet light. He found, however, that there was no virtue in the green coloration of the nickel carbonate, inasmuch as equally good results were given by the pink cobalt carbonate. Perhaps Baly's results may be summed up by a few quotations from his paper.

" To sum up the results, so far as they have been described, it has been found possible in the laboratory to produce carbohydrates directly from carbonic acid by a process which is physically similar to that of the living plant. The essential difficulty in our understanding of the natural photosynthesis has been solved, namely, the use of visible light as the agent in a process which the elementary laws of photochemistry taught

[12] Baly, E. C. C., Davies, J. B., Johnson, M. R., and Shanassy, H., The Photosynthesis of Naturally Occurring Compounds. I. The Action of Ultra-Violet Light on Carbonic Acid, *Proc. Roy. Soc.*, (A) 116 : 197–211 (1927).

[13] Baly, E. C. C., Stephen, W. E., and Hood, N. R., The Photosynthesis of Naturally Occurring Compounds. II. The Photosynthesis of Carbohydrates from Carbonic Acid by Means of Visible Light, *Proc. Roy. Soc.* (A) 116 : 212–219 (1927).

[14] Baly, E. C. C., and Davies, J. B., The Photosynthesis of Naturally Occurring Compounds. III. Photosynthesis *in vivo* and *in vitro*, *Proc. Roy. Soc.*, (A) 116 : 219–226 (1927).

[15] Baly, E. C. C., Photosynthesis, *Science*, 68 : 364–367 (1928).

us to believe could only be achieved by means of ultra-violet light. As so often happens the explanation when found is very simple. The great amount of energy required to convert the carbonic acid into carbohydrates is supplied to it in two portions, one by the surface and the other by the visible light.

" Nothing has as yet been said of the actual carbohydrates which have been photosynthesized in the laboratory. Although as yet our information is still meager, there is no doubt that the photosynthetic syrup is a mixture containing glucose or fructose, or both. There are also present more complex carbohydrates, which can be resolved to the simple sugars by the action of dilute acid. The analogy with the products of natural photosynthesis is too close to be passed by without comment.

" The similarity between the vital and the laboratory processes is not confined to the fact that the products from the two are the same. Botanists tell us that in the living plant the photosynthesis takes place on a surface, so also is a surface necessary in the laboratory. It has been found possible to compare the quantities of carbohydrates synthesized for equal areas exposed to light in the case of living leaves and the glass vessels of the laboratory. These quantities are about the same. Some plants produce more and others produce less than we are able to synthesize. This similarity may be emphasized, because surely Dame Nature in the living leaf has produced the best machine she could for her purpose of food production for her children of the vegetable kingdom.

" There is yet another striking feature which is common to the two, photosynthesis *in vivo* and *in vitro*. The light must not be too strong in either, for if it is too strong then harmful results at once supervene. This is due to the poisoning of the surface by the oxygen which is set free. In both cases this poisoning slowly rights itself, and in both the synthesis must not proceed at a greater rate than that of the recovery of the surface from its poisoning.

" In fine, so far as we have been able to carry the investigations, the processes in the living plant and in the laboratory show most striking resemblance, not only in the compounds which are formed, but in every feature which is characteristic of either of them."

Baly not only investigated the formation of carbohydrates from carbon dioxide and water but studied the second phase of the photosynthetic reaction, *i.e.*, the condensation of formaldehyde to sugars. In this instance, he irradiated an aqueous solution of formaldehyde in quartz with ultraviolet light, causing its condensation to a syrup. Several hundred grams of this syrup was prepared and has been studied by

Irvine and Francis.[16] These workers point out that hexose sugars are present in this syrup. Even a more striking result is their announcement that a very considerable part of the sugars which are present are aldoses. As we shall see later, the sugars which are formed from formaldehyde, polymerized by chemical agents such as the "formose" of Butlerow, are mainly ketoses. It would seem, therefore, that possibly Baly has been able to synthesize glucose or some similar aldose directly from formaldehyde.

The search for formaldehyde or other intermediate products in green leaves during the period of active photosynthesis has until very recently yielded entirely negative results. Numerous workers have announced from time to time their findings of formaldehyde in green leaves, only to have the evidence overthrown by later and more accurate investigations. Klein and Werner[17] have, however, recently announced the presence of formaldehyde as a constant product in leaves undergoing active photosynthesis. They have apparently eliminated the possibility that the formaldehyde which they obtained arose as a decomposition product of the organic materials present. They have made use of the new reagent, "dimedon" (dimethyl hydro-resorcinol), which condenses with formaldehyde to form an extremely insoluble and characteristic crystallization product. They observed the presence of formaldehyde regularly in leaves undergoing photosynthesis, whereas leaves which had been exposed to the dark for some time contained no formaldehyde, nor did chlorophyll extracts or leaves which had been macerated. Similarly, in experiments where leaves were exposed to the light in a carbon dioxide-free atmosphere, no formaldehyde was produced. Neither was it produced when leaves were treated with narcotics or poisons, such as phenylurethan, hydrocyanic acid, or sulfurous acid. They note that the concentration of formaldehyde remains at a very low level (8 to 15 mg. per 10 gms. of fresh leaves) which would indicate that if it is an intermediate product in photosynthesis, it is very rapidly condensed into sugars.

Numerous investigators have studied solutions of formaldehyde and other aldehydes as a source of energy for plant life. One of the more recent studies is that by Sabalitschka and Weidling[18] who note that *Elodea canadensis* can utilize formaldehyde in the dark, converting it

[16] Irvine, J. C., and Francis, G. V., Examination of Photosynthetic Sugars by the Methylation Method, *Ind. Eng. Chem.*, 16 : 1019–1020 (1924).

[17] Klein, G., and Werner, O., Formaldehyd als Zwischenprodukt bei der Kohlensäureassimilation, *Biochem. Z.*, 168 : 361–386 (1926).

[18] Sabalitschka, Th., and Weidling, H., Über die Ernährung von Pflanzen mit Aldehyden. VI. Polymerisation des Formaldehyds durch Elodea canadensis zu höheren Kohlehydraten, *Biochem. Z.*, 172 : 45–57 (1926).

into starch. Illumination does not appreciably alter this process. A concentration of 0.024 per cent formaldehyde gave the best results, more than that amount producing a toxic effect. The fact that it is utilized in the dark and transformed into starch is taken as evidence that it is a normal product of photosynthesis.

In a later paper[19] the same authors report studies on the assimilation of acetaldehyde by *Elodea canadensis*, and they find again that acetaldehyde is transformed into starch by the plants grown both in the dark and in the light. Figure 113 shows their results expressed in percentage of starch in 100 grams of dry material after the plants have been kept in the

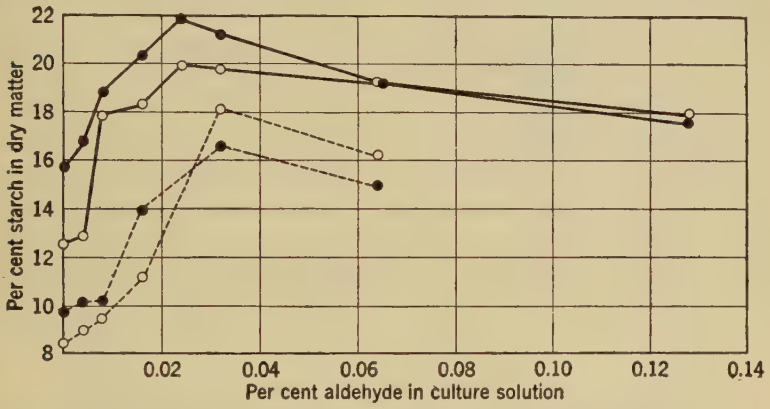


FIG. 113.—Showing that formaldehyde and acetaldehyde may serve as a source for starch formation by *Elodea canadensis*. Solid lines show formaldehyde; dotted lines, acetaldehyde data. Open circles, 10 days in the light; solid dots, 10 days in the dark. (Data of Sabalitschka.)

dark for a period of ten days in aldehyde solutions of different concentrations. A part of their data is also shown in Table LV.

From their experiments we must conclude that both formaldehyde and acetaldehyde may act as nutrients for green plants, both being capable of being transformed into starch and stored in the leaves. Accordingly, if Baly's experimental results are upheld by the future investigators, we would have a complete chain of evidence, from carbon dioxide and water to starch, in favor of the formaldehyde theory.

WHAT IS THE FIRST SUGAR FORMED IN THE PROCESS OF PHOTOSYNTHESIS?—Spoehr considers this problem at length and notes that the evidence is still inconclusive. The solution of this problem would greatly

[19] Sabalitschka, Th., and Weidling, H., Über die Ernährung von Pflanzen mit Aldehyden. VII. Erhöhung des Kohlehydratgehaltes von Elodea canadensis durch Acetaldehyd, *Biochem. Z.*, 176 : 210–224 (1926).

TABLE LV

Showing that *Elodea canadensis* is Able to Utilize Formadehyde and Acetaldehyde in the Dark, for Starch Formation
(Data of Sabalitschka)

	Formaldehyde Added per Liter of Solution,* Gms.	Starch Content (Dry Basis), Per Cent	Condition of the Plants	Acetaldehyde Added per Liter of Solution,† Gms.	Starch Content (Dry Basis), Per Cent	Condition of the Plants
Plants as collected (3 days in the light)	None	19.2	Good	None	20.3	Good
Plants (10 days in the dark)	None	15.7	Inferior	None	11.1	Somewhat inferior
	0.04	16.7	Good	0.01	11.2	Somewhat inferior
	0.08	18.4	Good	0.02	11.6	Somewhat inferior
	0.16	20.3	Good	0.04	13.2	Good
	0.24	21.9	Inferior	0.08	13.3	Good
	0.32	21.2	Injured	0.16	13.8	Good
	0.64	19.3	Greatly injured	0.32	18.4	Good
	1.28	17.5	Greatly injured	0.64	15.6	Injured
	....	....	.....	1.28	15.9	Greatly injured

* Sach's nutrient solution (0.1% salts), solution changed each day.
† Sach's nutrient solution (0.1% salts), solution changed each second day.

assist us in answering the questions as to the mechanism of photosynthesis. The data which are available, however, are very conflicting.

Thus, Brown and Morris,[20] from a study of the garden nasturtium, came to the conclusion that sucrose is the first product of photosynthesis, that it is a temporary reserve material which accumulates during the day, and that when it reaches a certain concentration, it is converted into starch. For translocation it is hydrolyzed into glucose and fructose. The fact that leaves of the nasturtium which have been actively photosynthesizing contain no glucose or fructose, is the basis for their argument that these sugars cannot be the primary products.

On the other hand, Priestley,[21] from a somewhat similar study, reaches the conclusion that sucrose is not the first sugar which is formed, and he concludes with the statement that the question must be regarded as still unsolved.

Spoehr concludes his discussion of this subject with the statement (p. 220), " In the present state of our knowledge glucose fits the theoretical requirements most adequately. Yet the fact cannot be entirely disregarded that the demonstration of glucose actually being the first sugar formed is still wanting."

The Energy Relations of Photosynthesis.—The two fundamental equations of living processes from the energy standpoint are usually written as,

$$6CO_2 + 6H_2O = C_6H_{12}O_6 + 6O_2 \quad \text{(photosynthesis)}$$

and

$$C_6H_{12}O_6 + 6O_6 = 6CO_2 + 6H_2O \quad \text{(respiration)}$$

In writing these chemical equations, the most significant part of the equations, insofar as vital processes are concerned, has been omitted, *i.e., the energy relationships.* The equations when correctly written should be,

$$6CO_2 + 6H_2O + 677.2 \text{ Cal.} = C_6H_{12}O_6 + 6O_2 \quad \text{(photosynthesis)}$$

and

$$C_6H_{12}O_6 + 6O_2 = 6CO_2 + 6H_2O + 677.2 \text{ Cal.} \quad \text{(respiration)}$$

It is through photosynthesis that the energy of sunlight is fixed and through oxidation that this fixed energy is released for the vital processes.

[20] Brown, H. T., and Morris, G. H., A Contribution to the Chemistry and Physiology of Foliage Leaves, *J. Chem. Soc.*, London, 63 : 604–677 (1893).

[21] Priestley, J. H., The First Sugar of Photosynthesis and the Rôle of Cane Sugar in the Plant, *New Phytologist*, 23 : 255–265 (1924).

It is generally stated that the efficiency of photosynthesis is extremely low. Warburg and Negelein[22] note that the efficiency of photosynthesis for alga, *Chlorella*, ranges from 59 to 63.5 per cent on the hypothesis that glucose is the primary product of photosynthesis. Adams[23] suggests that perhaps the reaction may be written,

$$6[CO_2 + 3H_2O = 2H_2O_2 + HCHO]$$

and on this basis, accounting for the energy in the formation of hydrogen peroxide, he calculates an efficiency of 98.6 per cent of the absorbed light.

Transeau[24] has summarized the energy relationships in a very striking manner. He selected an acre of corn in environmental conditions of north central Illinois and prepared a " budget " of the energy of the sunlight falling on that acre during the growing period and the fixation of the energy by the corn plant.

Taking a growing season of 100 days with 10,000 corn plants to the acre, and assuming a yield of corn of 100 bushels with a dry weight of 2160 kilograms per acre, the total dry weight of the 10,000 plants at maturity is approximately 6000 kilograms, 322 kilograms of which is mineral matter, leaving a residue of 5678 kilograms of organic matter of which 2675 kilograms is carbon. This amount of carbon would be equivalent to 6687 kilograms of glucose. This then is the amount of primary sugar equivalent to the carbon accumulated by the mature plant.

Transeau then notes that at maturity only a part of the carbon formed by synthesis, remains. A part has been lost by the plant in the process of respiration. He estimates respiration to release an amount of carbon dioxide per day equivalent to 1 per cent of the dry weight of the plant. The average dry weight for the season Transeau takes as one-half of the total weight, *i.e.*, 3000 kilograms. There would accordingly be 3000 kilograms of carbon dioxide, equivalent to 818 kilograms of carbon lost by respiration during the growing season. The glucose equivalent of the carbon dioxide respired for the entire acre, he calculates to be 2045 kilograms. He then adds this amount of glucose to the glucose equivalent of the carbon in the plant at maturity, giving the total glucose manufactured as 8732 kilograms. Inasmuch as it requires

[22] Warburg, O., and Negelein, E., Über den Einfluss der Wellenlänge auf den Energieumsatz bei der Kohlensäureassimilation, *Z. physik. Chem.*, 106 : 191–218 (1923).

[23] Adams, E. Q., The Efficiency of Photosynthesis by *Chlorella*, *J. Am. Chem. Soc.*, 48 : 292–294 (1926).

[24] Transeau, E. N., The Accumulation of Energy by Plants, *Ohio J. Sci.*, 26 : 1–10 (1926).

energy equivalent to 3760 Calories to produce 1 kilogram of glucose, he notes that it requires not far from 33,000,000 Calories to produce the entire photosynthetic product. The summation of the energy consumed in photosynthesis is shown in Table LVI.

TABLE LVI

Energy Consumed in Photosynthesis

(One acre of *Zea mays*, growing season of 100 days)

(Data of Transeau)

Glucose equivalent of accumulated carbon	6687 kg.
Glucose equivalent of carbon oxidized	2045 kg.
Total glucose manufactured	8732 kg.
Energy required to produce 1 kg. glucose	3760 Cal.
Total energy consumed in photosynthesis	33 million Cal.

Transeau then proceeds to estimate the efficiency of photosynthesis, noting that the total energy of sunlight falling on an acre of ground during the growing season is approximately 2043 million Calories. Of this enormous amount, only 1.6 per cent is accounted for in Table LVI as energy consumed in photosynthesis. He notes, however, that only approximately 20 per cent of the light of the spectrum is efficient for photosynthesis. On this basis, however, the efficiency of the photosynthetic process would be only 8 per cent. Facts, such as this, have been widely quoted to illustrate the inefficiency of the photosynthetic process.

Transeau, however, proceeds to note that there is another source of energy lost to the plant, *i.e.*, transpiration. Under the Illinois conditions which were studied, approximately 276 kilograms of water are evaporated during the growing season for every kilogram gained in dry weight. The total weight of water lost by transpiration is, therefore, approximately 1,500,000 kilograms which would be equivalent to 408,000 gallons or sufficient water to cover the acre to a depth of 15 inches. The energy required to evaporate 1 kilogram of water at the average temperature of the growing season is approximately 593 Calories, or the total energy consumed in the process of transpiration is approximately 910,000,000 Calories, equal to 44.5 per cent of the total energy of the sun falling upon the acre.

Respiration again releases a part of the energy rendered potential in photosynthesis. We have already noted that 2045 kilograms of glucose are oxidized. This results in the liberation of 7,700,000 Calories, or almost one-fourth of the energy absorbed in the process of photosynthe-

sis. Transeau notes that if we assume that photosynthesis goes on for twelve hours during the day, and respiration twenty-four hours each day, the average rate of photosynthesis must be about 8 times as great as is the rate of respiration.

Transeau further notes that for a very considerable part of the growing season, the plants do not completely shade the ground. At the beginning of the growing season, the plants are extremely small, most of the acre is bare, and the energy falling upon this bare earth ought not to be charged against the efficiency of the photosynthetic process. It is only late in the growing season that the plants become sufficiently developed to completely shade the ground and thus intercept all of the light energy falling upon the acre.

Table LVII shows the summary budget presented by Transeau. It will be noted that the acre of corn utilized 46 per cent of the energy falling upon that acre, and the environment, *i.e.*, the air, the bare ground, etc., used up 54 per cent. If we assume, as seems reasonable, that the corn plant shaded the surface of the soil completely for only one-half of the time, Transeau's budget would account for a very high efficiency of the utilization of solar energy.

TABLE LVII

Summary of Energy Budget of One Acre of Corn During Growing Season

Total radiant energy available	2043 million Cal.
Used in photosynthesis	33 million Cal.
Used in transpiration	910 million Cal.
Total energy consumed	943 million Cal.
Energy not directly used by the plants	1100 million Cal.
Energy released by respiration	8 million Cal.
Of the available radiant energy, 100-bushel-corn uses about	46%
The environment takes up about	54%

The striking conclusions of Transeau's calculations are that the evaporation of water from an acre of growing corn consumes about 45 per cent of the available radiant energy, that of the carbohydrates synthesized approximately 23.4 per cent is again utilized by the plant for purposes of respiration, that the total energy content of the dry matter of the corn plant at maturity is equal to only 1.6 per cent of the energy falling upon the area during the growing period, and, if we consider only the grain, we have returned to us less than 0.5 per cent of the total radiant energy.

Transeau believes that corn is probably the most efficient of our temperate-zone plants and that very few of the crop plants in the tem-

perate regions can equal corn in calories of energy per acre. He further points out that the suggestion has been made that when the liquid fuels, such as petroleum and gasoline are gone, they may be replaced by alcohol made from plants, and notes that to substitute the energy of alcohol for the energy which is at the present time being used in the United States in the form of gasoline would require more corn than is now being grown in the United States.

Shull[25] has made a further contribution to the question of the energy relationships. In all of the earlier calculations on the efficiency of photosynthesis, it has been assumed that the light falling upon a leaf is all potentially available. Shull measured by means of a spectrophotometer the reflection of light from the surface of leaves. The percentage of reflection was usually low in the blue and red regions and highest in the green, the curve of reflection rising to a maximum at 540 to 560 $m\mu$. The lowest reflection was found in very dark green leaves, such as those of *Syringa vulgaris*, where the reflection was only 6 to 8 per cent, ordinary green leaves reflecting from 10 to 12 per cent. Spring verdure has a much higher reflection which may reach 20 per cent or more, while albino leaves and those which are pubescent, such as *Populus alba*, may reflect from 30 to 52 per cent of the incident light. It would seem that we should not charge the chloroplast with inefficiency in the photosynthetic mechanism when a very considerable proportion of the light never reaches the chloroplasts.

Classification of the Carbohydrates

I. *Simple Sugars or Monosaccharides.*—These may be defined as aldehyde or ketone alcohols of the aliphatic series, the molecule of which contains one carbonyl group and one or more alcohol groups, one of the latter always being adjacent to the carbonyl group. According to this definition formaldehyde is not a carbohydrate.

A. The *aldose* sugars contain a potential aldehyde group.

B. The *ketose* sugars contain a potential ketone group.

The sugars may be further subdivided according to the number of carbon atoms.

1. Monoses, CH_2O

   Formaldehyde, first homologue of series *but not a carbohydrate*

[25] Shull, C. A., Reflection of Light from the Surfaces of Leaves, *Science*, 67 : 107–108 (1928).

2. Dioses, $C_2H_4O_2$
   Glycolaldehyde

3. Trioses, $C_3H_6O_3$
   a. Aldotriose
      d- and *l*-glycerose (glyceric aldehyde)
   b. Ketotriose
      Dioxyacetone

4. Tetroses, $C_4H_8O_4$
   a. Aldotetroses (4 possible isomers)
      d- and *l*-erythrose
      d- and *l*-threose
   b. Ketotetroses
      Erythrulose
   c. Hydroxymethyltetrose
      Apiose (β-hydroxymethyltetrose from the glucoside, apiin)

5. Pentoses, $C_5H_{10}O_5$
   a. Aldopentoses (8 possible isomers)
      d- and *l*-arabinose
      d- and *l*-xylose
      d- and *l*-ribose
      d- and *l*-lyxose
   b. Methylpentoses (One of the hydrogen atoms of the primary alcohol group in an aldopentose is replaced by a methyl group.
      Rhamnose (component of many vegetable glucosides)
      Fucose (Fucosan found in seaweed of Fucus variety[26])
      Rhodeose (occurs in certain glucosides)
   c. Methoxymethyl pentose
      Digitalose ($C_7H_{14}O_5$, in the glucoside, digitalin)
   d. Methylthio pentose ($C_6H_{12}O_4S$, from yeast, cf. p. 398)
   e. Ketopentoses (4 possible isomers; none have been isolated in pure form.)

6. Hexoses, $C_6H_{12}O_6$
   a. Aldohexoses (16 possible isomers, 14 known)
      d- and *l*-mannose
      d- and *l*-glucose
      d- and *l*-idose

[26] Clark, E. P., The Structure of Fucose, *J. Biol. Chem.*, 54 : 65–73 (1922).

d- and *l*-gulose
d- and *l*-galactose, "cerebrose" (Thudichum)
d- and *l*-talose
d-allose
d-altrose

b. Ketohexoses (8 possible isomers, 5 known)
d- and *l*-fructose
d- and *l*-sorbose
d-tagatose

7. Heptoses, $C_7H_{14}O_7$
   a. Aldoheptoses (3 known)
   Glucoheptose
   Mannoheptose
   Galactoheptose
   b. Ketoheptoses (2 known)
   Sedoheptose (in all probability a ketose, cf. La Forge and Hudson[27])

   Perseulose (from the avocado, *Persea gratissima*)

8. Octoses, $C_8H_{16}O_8$
   a. Aldooctoses (3 known)
   Glucooctose
   Mannoctose
   Galactooctose

9. Nonoses, $C_9H_{18}O_9$
   a. Aldononoses (2 known)
   Gluconononose
   Mannononose

10. Decoses, $C_{10}H_{20}O_{10}$
   a. Aldodecoses
   Glucodecose

The pentose and hexose sugars are the most important of the simple sugars.

II. *Compound Sugars.*—By the condensation of 2, 3, or 4 molecules of the monosaccharides, disaccharides, trisaccharides, and tetrasaccharides are formed. In these condensations one molecule less of water is eliminated than the number of reacting sugars.

[27] La Forge, F. B., and Hudson, C. S., Sedoheptose, A New Sugar from *Sedum spectabile*. I., *J. Biol. Chem.*, 30: 61–77 (1927).

$2C_6H_{12}O_6 - H_2O = C_{12}H_{22}O_{11}$ (disaccharide)
$3C_6H_{12}O_6 - 2H_2O = C_{18}H_{32}O_{16}$ (trisaccharide)
$4C_6H_{12}O_6 - 3H_2O = C_{24}H_{42}O_{21}$ (tetrasaccharide)

1. Disaccharides
   - *a.* Dipentose saccharides, $C_5H_9O_4—O—C_5H_9O_4$
     Diarabinose, $C_{10}H_{18}O_9$
   - *b.* Pentose-hexose saccharides, $C_5H_9O_4—O—C_6H_{11}O_5$
     Glucoapiose, $C_{11}H_{20}O_{10}$ (in the glucoside, apiin)
     Galactoarabinose, $C_{11}H_{20}O_{10}$ (synthetic)
     Glucose-arabinoside
     Vicianose (in *Vicia angustifolia*)
     Glucose xyloside
     Primeverose (in *Primula officinalis*)
   - *c.* Methylpentose-hexose saccharides,

     $$CH_3—C_5H_8O_4—O—C_6H_{11}O_5$$

     Glucorhamnoside (in the glucoside convolvulin)
   - *d.* Dihexose saccharides, $C_6H_{11}O_5—O—C_6H_{11}O_5$ or $C_{12}H_{22}O_{11}$
     Sucrose, maltose, and lactose are the most important of the higher saccharides.

Type I. Aldehyde group potentially functional

Maltose	α Glucose α glucoside
Lactose	β Glucose β galactoside
Isolactose	Glucose ? galactoside
Melibiose	β Glucose α galactoside
Turanose	Glucose ? fructoside
Gentiobiose or isomaltose	β Glucose β glucoside
Cellose or cellobiose	β Glucose β glucoside

Type II. Aldehyde group not functional (no reducing properties)

Sucrose	γ Fructose α glucoside
Trehalose	α Glucose α glucoside
Isotrehalose	β Glucose β glucoside

2. Trisaccharides, $C_{18}H_{32}O_{16}$

Type I. Reducing

Methylpentose-hexose saccharides
Rhaminose (glucose + rhamnose + rhamnose)

Robinose [galactose + rhamnose + rhamnose (from the glucoside, robinine)]

Trihexose saccharide

Mannotriose saccharide (glucose + galactose + galactose)

Type II. Non-reducing

Trihexose saccharides

Raffinose (galactose + glucose + fructose)

Melezitose (glucose + glucose + fructose)

Gentianose (fructose + glucose + glucose)

3. Tetrasaccharides, $C_{24}H_{42}O_{21}$

Type II, Non-Reducing

Tetrahexose saccharides

Stachyose (fructose + glucose + galactose + galactose)

4. Polysaccharides or non-sugars.—Formed by the condensation of an indefinite number of monosaccharide molecules

a. Pentosans $(C_5H_8O_4)_x$

Araban

Xylan

b. Methyl pentosans

Rhamnan

c. Hexosans $(C_6H_{10}O_5)_x$

Dextrosans

Dextrin

Starch

True or normal cellulose

Lichenin (a constituent of many mosses and lichens)

Dextran (a mucilaginous substance secreted by many bacteria)

Glycogen

Levulosans

Inulin

Mannosans

Mannan

Galactosans

Galactan

d. Mixed pentosans

Gums

Mucilages

Hemicelluloses
Pectins

e. Mixed hexosans
Compound celluloses
Ligno-celluloses (non-cellulose constituent-lignin)
Pecto-cellulose (non-cellulose constituents—pectic substances)
Adipo-cellulose (non-cellulose constituents—fatty substances, as suberin and cutin)

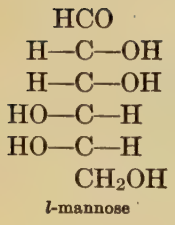
5. Other carbohydrate linkings

a. The amino sugars
Aminoglucose or glucosamine
Chitin

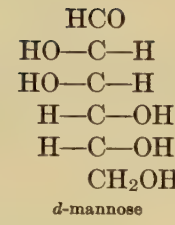
b. The cycloses
Inositols.—They are isomeric with the hexoses.
Phytin.—Inositol (hexahydroxyhexahydrobenzene) exists in nature in combination with phosphoric acid as phytin, the principal phosphorus compound of vegetable seeds. Inositol is one of the "bios" compounds.

Structural Formulae

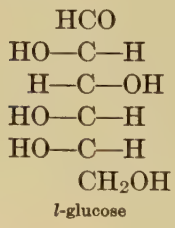
Aldohexoses



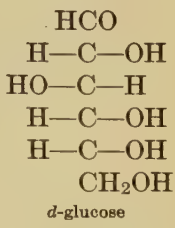
l-mannose



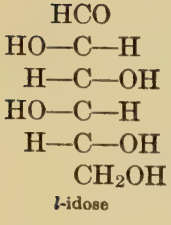
d-mannose



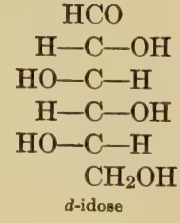
l-glucose



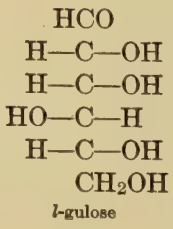
d-glucose



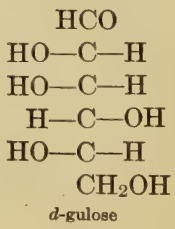
l-idose



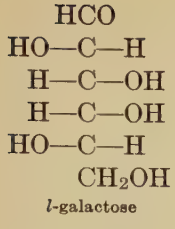
d-idose



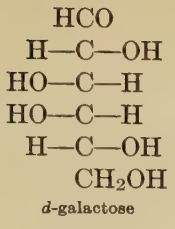
l-gulose



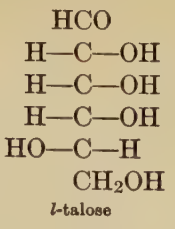
d-gulose



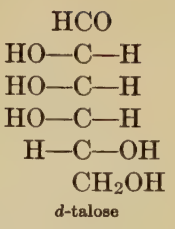
l-galactose



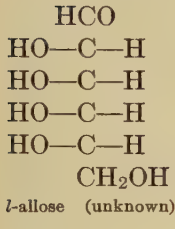
d-galactose



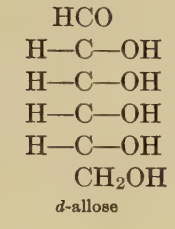
l-talose



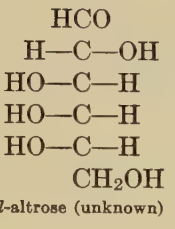
d-talose



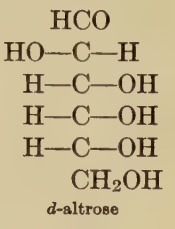
l-allose (unknown)



d-allose



l-altrose (unknown)



d-altrose

CHAPTER XXII

THE STRUCTURE OF THE MONOSACCHARIDE MOLECULE

THE CHEMICAL STRUCTURE OF GLUCOSE.—The chemical configuration of the glucose molecule has been derived from a series of organic studies

1. By elementary analysis and molecular weight determinations, the formula has been shown to be $C_6H_{12}O_6$.

2. Reduction with hydriodic acid yields *n*-secondary-hexyliodide, $CH_3—CH_2—CHI—CH_2—CH_2—CH_3$, which is a derivative of *n*-hexane. All aldohexoses contain the *n*-hexane chain. Therefore, the straight-chain formula for the carbon portion of the molecule is proven.

3. The glucose molecule contains 5 hydroxyl groups, inasmuch as it forms a penta-acetyl glucose when treated with acetyl chloride or acetyl anhydride.

$$R—OH + \begin{matrix} CH_3—CO \\ \\ CH_3—CO \end{matrix}\!\!>O = R—O—CO—CH_3 + CH_3—COOH$$

$$R—OH + CH_3—CO—Cl = R—O—CO—CH_3 + HCl$$

The number of acetyl groups in a compound can be determined by ascertaining the *acetyl number* which depends upon the reaction,

$$R—O—CO—CH_3 + KOH = R—OH + CH_3—CO—OK$$

one equivalent of KOH being utilized for each acetyl group in a gram molecule of the acetyl derivative.

4. Glucose contains an aldehyde or a potential aldehyde group, inasmuch as it adds hydrocyanic acid to form a cyanhydrin; it reduces alkaline copper solution; it forms oximes with hydroxylamine, and hydrazones (and osazones) with hydrazines.

5. On oxidation, glucose yields a monobasic acid (gluconic acid) containing 6 carbon atoms. Therefore, the carbonyl group is on one of the terminal carbon atoms. Fructose under the same conditions of oxidation breaks up, yielding, among other products, trihydroxy-butyric acid.

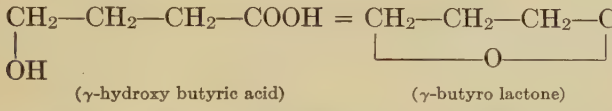
6. The stability of the molecule is evidence that not more than one hydroxyl group is on any one carbon atom.

Since there are 5 hydroxyl groups and a carbonyl group on a terminal carbon atom this yields

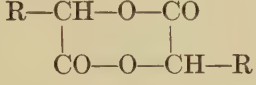
$$CH_2OH—CHOH—CHOH—CHOH—CHOH—CHO,$$

as the formula of glucose. This formula, however, does not give us any information as to the space configuration of the asymmetric carbon atoms. These space configurations will be considered later. All of the hexoses have the same straight-chain carbon formula as noted above, when we consider the aldehyde group as being free.

THE CLOSED-CHAIN FORMULA OF THE MONOSACCHARIDES.—Certain hydroxy compounds readily lose water or undergo rearrangements, with the formation of ring structures. Thus, the γ-hydroxy acids lose water to form γ-lactones or anhydrides, the ring containing 4 carbon atoms and 1 oxygen atom.



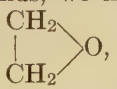
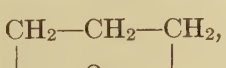
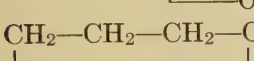
The name, *lactone*, is applied only to rings produced by internal anhydride formation from an hydroxy acid. Thus, two molecules of α-hydroxy acids usually combine to form an anhydride of the type,



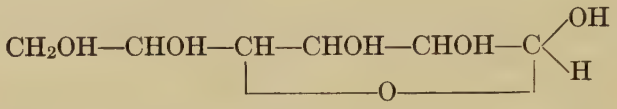
β-hydroxy acids lose water to form unsaturated compounds,

$$CH_3—CHOH—CH_2—COOH \rightarrow CH_3—CH = CH—COOH,$$

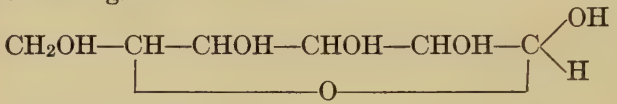
and γ and δ acids to form lactones.

When oxygen rings are formed from organic compounds other than acids, they are known as *oxides*. Thus, we may have an α-oxide or ethylene-oxide ring with the formula,  , a β-oxide or propylene oxide ring,  , a γ-oxide or butylene-oxide ring,  , or a δ-oxide or amylene-oxide ring, , etc.

Tollens, in 1883, proposed a closed-ring formula for glucose containing 4 carbon atoms and an oxygen atom. This formula was generally adopted, inasmuch as it appeared to represent more accurately the reactions of the carbohydrates than did the straight-chain formula. An oxide containing 4 carbon atoms would be a γ-oxide, and in the older literature the closed-ring formula of the aldohexoses is figured as a γ-oxide or butylene-oxide ring.

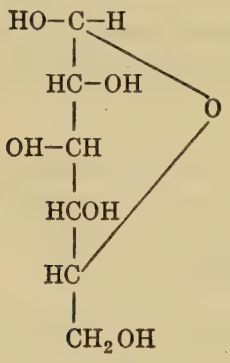


Recent researches have indicated very clearly that in most instances the ring is not a γ-oxide or butylene-oxide ring but rather a δ-oxide or amylene-oxide ring.

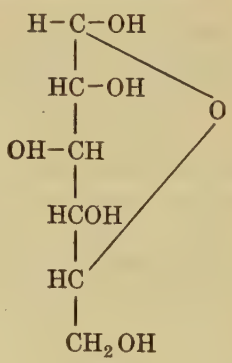


This point will be referred to again.

The straight-chain formula provides for only one modification of *d*-glucose, whereas the oxide formula allows for two sugars having the same space relations as *d*-glucose, due to the fact that there is a possibility of isomerism in the terminal carbon atom of the oxide ring, thus introducing a new asymmetric carbon atom. *Two forms of d-glucose have actually been isolated and their formulae may be written*:



α-Glucose

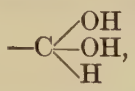


β-Glucose

β-glucose, for convenience, has been designated as the " cis " form, in which the —OH of the potential carbonyl group is on the same side of the molecule as the oxygen ring, whereas the α form may be regarded as the " trans " form, in which the —OH of the potential aldehyde group is

on the opposite side of the molecule from the oxygen ring (cf. Levene and Sobotka).[1]

The aldehyde reactions are explained by a rupture of the oxide ring and the formation by the addition of water, first of an aldehydrole,



then by a loss of water from the aldehydrole to form an aldehyde group. The oxide ring structure thus allows for 5 asymmetric carbon atoms in place of the 4 of the straight-chain formula, and for 32 instead of 16 isomeric aldohexoses.

The actual arrangement in space of the atoms in a glucose molecule can be represented only very imperfectly by a plane diagram. Sponsler and Dore[2] have prepared certain three-dimensional models in order to illustrate the actual space relations of the atoms. Figure 114, taken from their paper, shows the space relations of the carbon, hydrogen, and oxygen atoms as they probably exist in the amylene-oxide form of *β-d*-glucose. A comparison of this figure with the plane graphic formula of the δ-oxide form of *β-d*-glucose illustrates the inadequacy of the plane formula.

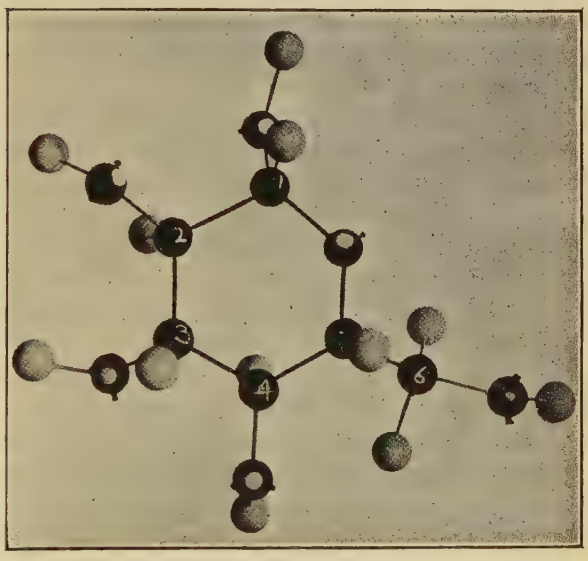


FIG. 114.—The space configuration of a three-dimensional model representing the amylene oxide form of *β d*-glucose. (After Sponsler and Dore.)

Within the last few years we have seen a drastic revision of the

[1] Levene, P. A., and Sobotka, H. A., On the *α*- and *β*-Forms of Sugars and of Sugar Derivatives, *Science*, 63 : 73–74 (1926).

[2] Sponsler, O. L., and Dore, W. H., The Structure of Ramie Cellulose as Derived from X-Ray Data, Colloid Symposium Monograph, Vol. IV, pp. 174–202 (1926).

structural formulae of most of the simple sugars, due primarily to the work of Hirst, Haworth, and Irvine, and their coworkers. Hirst and Purves,[3] and Hirst and Robertson[4] have shown that the pentose sugars, xylose and arabinose, have the δ-oxide or amylene-oxide structure. Hirst and Macbeth[5] have shown that rhamnose and its stable derivatives have the amylene-oxide structure. Charlton, Haworth, and Peat[6] note that, " It is clear that the normal sugars (xylose and arabinose) are amylene-oxidic in structure and the labile or γ-forms are butylene-oxides. The related lactones are therefore δ-lactones for the normal sugars and γ-lactones for the labile or γ-sugars." . . . " All the commonly occurring aldoses are therefore shown to exist normally as amylene- or 1 : 5-oxide forms, or ..... as δ-oxides."[7] Charlton, Haworth, and Peat note from their work and from previous work that all of the formulae of lactose, cellobiose, maltose, gentiobiose, melibiose, and sucrose must be revised. Hirst,[8] in a study of the structure of glucose, concludes that it likewise is the amylene-oxide form (cf. also Drew and Haworth).[9]

Besides the α- and β-forms of sugars there exists a γ-form which cannot be isolated directly, because of its great reactivity. Derivatives, however, may be prepared, and such derivatives as tetramethyl-γ-*d*-glucose, trimethyl-γ-*d*-glucose, tetramethyl-γ-*d*-galactose, tetramethyl-γ-*d*-mannose, tetramethyl-γ-*d*-fructose, and trimethyl-γ-*d*-fructose have been prepared and isolated. *All undergo oxidation with great ease, e.g.*, trimethyl-γ-*d*-glucose reduces Fehling's solution instantly at room temperature. At ordinary temperatures they form glucosides almost instantaneously, whereas it is necessary to heat the reaction mixture for sixty hours at 100° to form α-methyl glucoside.

Apparently γ-glucose, which can be taken as a representative of the γ-sugars, differs from α- and β-glucose by not having the hexaphane

[3] Hirst, E. L., and Purves, C. B., The Structure of the Normal Monosaccharides, Pt. I., Xylose, *J. Chem. Soc.*, London, 123 : 1352–1360 (1923).

[4] Hirst, E. L., and Robertson, G. J., The Constitution of the Normal Monosaccharides, Pt. II., Arabinose, *J. Chem. Soc.*, London, 127 : 358–364 (1925).

[5] Hirst, E. L., and Macbeth, A. K., The Structure of the Normal Monosaccharides, Pt. III., Rhamnose, *J. Chem. Soc.*, London, Jan. 1926, 22–26.

[6] Charlton, W., Haworth, W. N., and Peat, S., A Revision of the Structural Formula of Glucose, *J. Chem. Soc.*, London, Jan., 1926, 89–101.

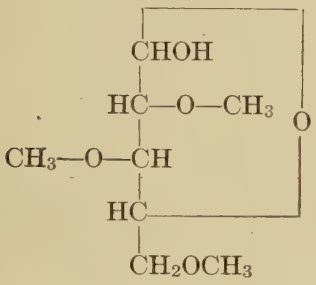
[7] Note that the conclusions reached in this paper as to the structure of the ketose sugars are not correct (cf. Haworth, W. N., and Hirst, E. L., The Structure of Fructose, γ-Fructose, and Sucrose, *J. Chem. Soc.*, London, July, 1926, 1858–1868.)

[8] Hirst, E. L., The Structure of the Normal Monosaccharides, Pt. IV., Glucose, *J. Chem. Soc.*, London, Feb., 1926, 350–357.

[9] Drew, H. D. K., and Haworth, W. N., A Critical Study of Ring Structure in the Sugar Group, *J. Chem. Soc.*, London, Sept., 1926, 2303–2310.

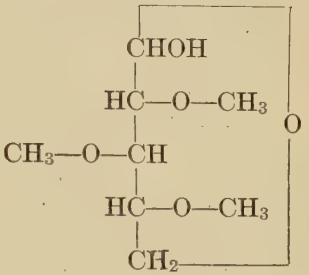
ring. It has been suggested that it may contain an ethylene-oxide or 3-membered ring. Charlton, Haworth, and Peat[10] state that the evidence appears to be practically conclusive that the γ-sugars have the γ-oxide ring (cf. also Irvine).[11, 12]

In the case of the pentoses, Haworth and Westgarth[13] find the same generalization to hold, *i.e.*, that the ordinary form of xylose is the amylene-oxide structure, whereas the γ-form contains the butylene-oxide ring. They prepared trimethyl-γ-xylose and the trimethyl derivative of ordinary xylose, having the following structures and physical and chemical properties:



Trimethyl-γ-xylose

Syrup, b.p. 110°/0.04 mm.
$[\alpha]_D = +24.7° \rightarrow 31.2°$.
Reduces cold permanganate.
Combines with acid methyl alcohol.



Ordinary trimethyl-xylose

Crystalline, m.p. 87°–90°.
$[\alpha]_D = +74° \rightarrow 21°$.
No action with permanganate.
Slow combination with acid methyl alcohol.

It is impracticable to note the physical and chemical properties of the various derivatives which have been prepared, but the two inserted above may be regarded as typical.

Tetramethyl γ-galactose readily undergoes spontaneous condensation with itself at room temperature to form a non-reducing disaccharide. Irvine suggests that in this case the γ-form possibly possesses the propylene-oxide ring, although the ethylene-oxide ring is not excluded. Normal fructose apparently contains the amylene-oxide or δ-oxide ring.

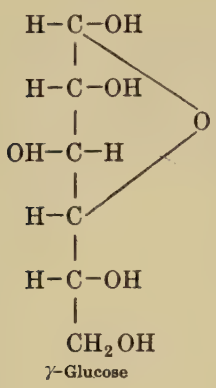
[10] Charlton, W., Haworth, W. N., and Peat, S., A Revision of the Structural Formula of Glucose, *J. Chem. Soc.*, London, Jan., 1926, 89–101.

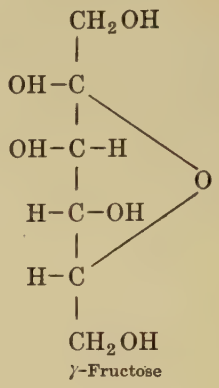
[11] Irvine, J. C., The Biological and Chemical Significance of Gamma Sugars, *Ind. Eng. Chem.*, 15 : 1162–1164 (1923).

[12] Irvine, J. C., Some Constitutional Problems of Carbohydrate Chemistry, *J. Chem. Soc.*, London, 123 : 898–921 (1923).

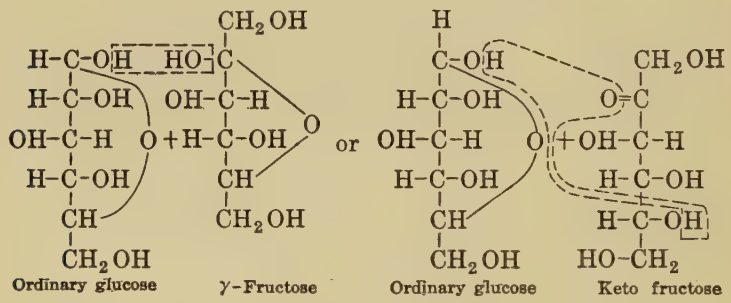
[13] Haworth, W. N., and Westgarth, G. C., Synthesis of Derivatives of γ-Xylose, *J. Chem. Soc.*, April, 1926, 880–887.

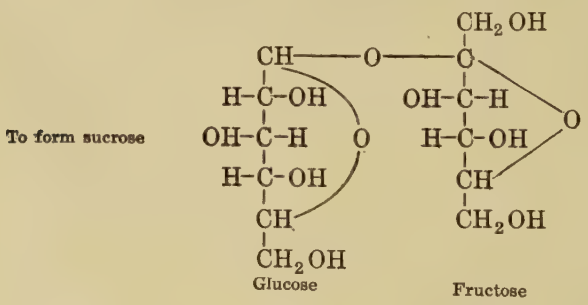
Haworth and Hirst[14] note that this appears to be true for all of the ketoses. Accordingly, the following formulae appear to hold for γ-glucose and γ-fructose:





Sucrose may be regarded as having been formed from the condensation of ordinary glucose and γ-fructose by the elimination of water, or by the condensation of ordinary glucose with the keto form of fructose, as shown in the following formulae:



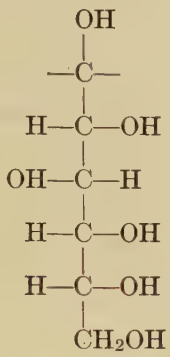


[14] Haworth, W. N., and Hirst, E. L., The Structure of Fructose, γ-Fructose, and Sucrose, *J. Chem. Soc.*, London, July, 1926, 1858–1868.

It will be noted in the last case that a γ-sugar residue is formed from a condensation involving no γ-sugars.

Irvine suggested at one time that perhaps the γ-fructose was actually the free keto-form and the γ-glucose was actually the free aldo-form with no ring structures.

Recently Clifton[15] has suggested that the active form of glucose is neither the aldehyde form nor does it contain an oxide ring, instead suggesting that it may be a form of glucose, containing divalent carbon, derived from ordinary glucose by an opening of the oxide ring and having the formula,



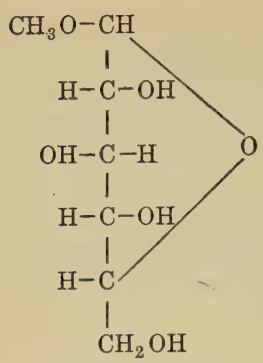
Perhaps the reactions of the sugars in nature, when starch and the disaccharides are formed, are due to γ-sugars condensing with each other, rather than to reactions involving the more stable α- and β-forms.

Hudson,[16] from a study of optical rotations, agrees with the English workers that the stable forms of xylose, arabinose, and galactose possess the δ-oxide rings. He does not, however, agree to the amylene-oxide ring for glucose, and insists that it must be a butylene-oxide ring. He suggests that the γ-sugars contain an oxide ring involving the first and third carbon atoms.

The Methyl Glucosides.—There are two isomeric methyl glucosides derived from the α- and β-modifications of glucose. These are analogous to the naturally-occurring glucosides. Fischer prepared these glucosides in 1893 by dissolving glucose in cold methyl alcohol and saturating the solution with dry hydrogen chloride. Their formulae may be represented as,

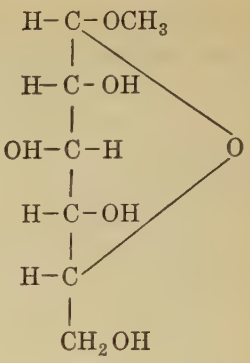
[15] Clifton, C. E., Potentiometric Studies of Sugar Oxidation, A Determination of Active Glucose, Ph.D. thesis, University of Minnesota, Dec., 1928.

[16] Hudson, C. S., Relations between Rotatory Power and Structure in the Sugar Group. XIV. The Determination of Ring Structures in the Glucose, Mannose and Rhamnose Series, *J. Am. Chem. Soc.*, 48 : 1434–1443 (1926).



α-Methyl glucoside

and



β-Methyl glucoside

A study of these methyl glucosides has given added assurance of the evidence of α- and β-forms of glucose in the ordinary glucose of the chemical laboratory.

The methyl glucosides never behave as aldehydes. The aldehyde group is entirely masked. Their rotatory power is the same in a freshly prepared solution as in an older solution. This is not true of glucose (*vide infra*) where the α- and β-forms are in equilibrium in different amounts under different conditions of temperature, concentration, etc.

The methyl glucosides may in turn be hydrolyzed to glucose and methyl alcohol.[17] However, the same enzyme will not hydrolyze both, *i.e.*, we have specific enzymes for hydrolyzing the α- and β-glucosides. α-Methyl glucosides are hydrolyzed by the enzyme, *maltase*, the enzyme that splits the disaccharide, maltose, and the β-methyl glucosides are hydrolyzed by the enzyme, *emulsin*, the enzyme that hydrolyzes the naturally-occurring glucosides. These enzymes act best at about 37° C. (time = several hours) and are more active hydrolyzing agents than are acids.

When solid anhydrous glucose (largely α-glucose) is used for the preparation of the methyl glucosides, the equilibrium mixture contains 77 per cent of the α-methyl glucoside and 23 per cent of the β-isomeride. To prepare the pure β-form, a biological method is used. Ordinary bakers' yeast contains maltase which will split the α-form, and zymase which will ferment the resulting glucose to CO_2 and ethyl alcohol. Consequently, if a mixture of α- and β-methyl glucosides is incubated with yeast, the α-methyl glucoside is split, and the glucose is fermented, leaving the β-methyl glucoside unaffected because no emulsin is present.

[17] Armstrong, E. F., Studies on Enzyme Action. 1. The Correlation of the Stereoisomeric α- and β-Glucosides with the Corresponding Glucoses, *J. Chem. Soc.*, London, 83 : 1305–1313 (1903).

MUTAROTATION OR MULTIROTATION.—A freshly prepared solution of an aldo or keto sugar changes its optical rotation upon standing; sometimes the rotation increases or it may decrease, depending upon the sugar being investigated. Eventually, however, a constant value is reached, the final reading depending upon the factors of concentration, temperature, and time.

With anhydrous glucose the rotation falls about 50 per cent. The hypothesis of two stereoisomeric forms of glucose is the only one that affords an adequate explanation. Lippmann,[18] in 1895, suggested that mutarotation in solution was due to a change from one oxide form to the other. At that time, only one anhydrous glucose was known, and the existence of isomers was purely hypothetical. In this and the following year (1895–96), Tanret[19] isolated some new forms of glucose, galactose, lactose, arabinose, and rhamnose, and separated what he thought were three distinct forms of *d*-glucose.

1. α-Glucose with an optical rotation of $+110°$, falling gradually to $+52.5°$.

2. β-Glucose with an optical rotation of $+19°$, rising gradually to $+52.5°$.

3. "γ-Glucose" with an optical rotation of $+52.5°$ which remained constant in solution.

Simon[20] suggested that α- and β-glucose were homologues of α- and β-methyl glucosides and that both contained a closed oxygen ring analogous to the glucosides whose structure had already been established.

Armstrong[21] proved definitely that the α- and β-isomers have the oxide structure. He showed that enzymes hydrolyze the α- and β-methyl glucosides to α- and β-glucose respectively, yielding sugars with (*a*) high or (*b*) low initial optical rotation, and that regardless of what the initial rotation is, the same equilibrium rotation is eventually reached. Armstrong gives the following values for optical rotation:

α-methyl glucoside $[\alpha]_D = +157°$.	α-glucose $[\alpha]_D = +105°$.
β-methyl glucoside $[\alpha]_D = -33°$.	β-glucose $[\alpha]_D = +22°$.

As a result of a later investigation Hudson concludes that α-glucose

[18] Lippmann, E. O. von, Die Chemie der Zuckerarten, Zweite Auflage, Braunschweig (1895).

[19] Tanret, C., Sur les modifications moléculaires du glucose, *Bull. Soc. chim.*, (3) 13 : 728–735 (1895); Sur les modifications moléculaires et la multirotation des sucres, 15 : 195–205, 349–361 (1896).

[20] Simon, L. J., Sur le constitution du glucose, *Compt. rend.*, 132 : 487–490 (1901).

[21] Armstrong, E. F., Studies on Enzyme Action. 1. The Correlation of the Stereoisomeric α- and β-Glucosides with the Corresponding Glucoses, *J. Chem. Soc.*, London, 83 : 1305–1313 (1903).

has an initial rotation of $[\alpha]_D = +110°$, and β-glucose has an initial rotation of $[\alpha]_D = +19°$.

The average rotation of the glucosides is $+62°$, and of the glucoses, $+63.5°$ ($64.5°$ if Hudson's values are taken), and Simon points out that this fact indicates a close relation in structure.

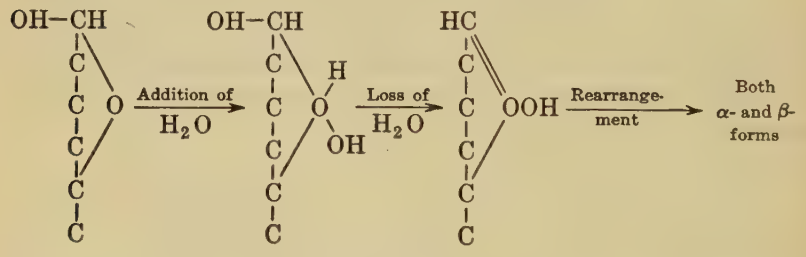
Tanret's "γ-glucose" proved to be the equilibrium mixture of the α- and β-modifications. Tanret, in 1905, accepted this view and calculated the equilibrium mixture of a 10 per cent glucose solution to contain 37 per cent of α-glucose and 63 per cent of β-glucose.

Glucose purified by crystallization from dilute methyl alcohol is invariably a mixture of α- and β-forms. Hudson and Dale[22] recommend acetic acid of different concentration to separate, by fractional crystallization, the α- and β-modifications.

Two explanations have been advanced to account for the mechanism of the isomeric shift of $\alpha \rightleftharpoons \beta$.

1. Lowry considers the formation of the aldehyde or its hydrate to be an intermediate step, thus opening the oxide chain.

2. Armstrong believes the oxide ring does not need to open. He considers that the first stage is the formation, by addition of water, of an *oxonium hydrate* which later loses water, a part of the elements of water coming from either the hydrogen or hydroxyl group attached to the terminal carbon atom, the intermediate compounds so formed rearranging to a mixture of the α- and β-forms. *The evidence in favor of the oxonium shift is now regarded as practically conclusive.*



Reactions of Sugars with Phenylhydrazine.—Sugars react with phenylhydrazine in dilute acetic acid solution to form *phenylhydrazones* as the first product.

$$R—CHO + C_6H_5—NH—NH_2 = R—CH=N—NH—C_6H_5 + H_2O.$$

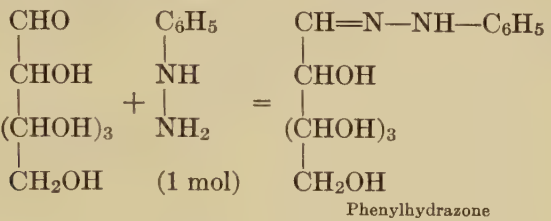
The phenylhydrazone can be decomposed and the original sugar regenerated by acting on the phenylhydrazone with benzaldehyde, resulting

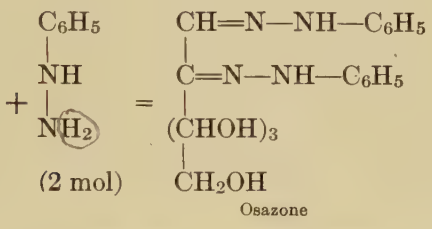
[22] Hudson, C. S., and Dale, J. K., Studies on the Forms of *d*-Glucose and Their Mutarotation, *J. Am. Chem. Soc.*, 39 : 320–328 (1917).

in forming the phenylhydrazone of benzaldehyde at the expense of the sugar phenylhydrazone.

Most of the phenylhydrazones of the sugars are easily soluble. Mannose is an exception, since it forms an almost insoluble phenylhydrazone, thus affording another striking illustration of the effect of molecular configuration on physical properties.

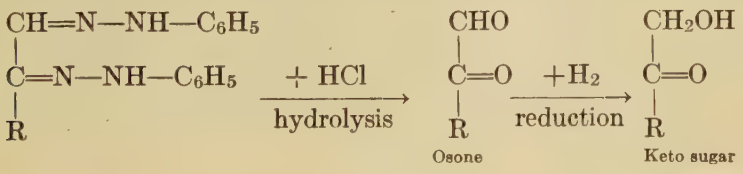
Excess of phenylhydrazine acts as an oxidizing agent toward the phenylhydrazone, converting the adjacent =CHOH group into a carbonyl (=C=O) group. This carbonyl group will then react with another molecule of phenylhydrazine to form an *osazone*.





Glucose, mannose, and fructose yield the same osazone because the asymmetry of the α-carbon is destroyed in the formation of the osazone. *This indicates that the remaining four carbon atoms have the same molecular configuration in all three sugars.* Glucosamine gives the same osazone as glucose, indicating that the $-NH_2$ group is on the α-carbon. Because of the fact that the asymmetry of the α-carbon atom is destroyed, one should not place too much confidence in the properties of an osazone in identifying a sugar.

When an osazone is treated with fuming hydrochloric acid, both phenylhydrazine groups are split off and an *osone* results. Here again, glucose, fructose, and mannose give the same osone. This is a colorless, strongly reducing syrup. On reducing this osone we obtain *d*-fructose.



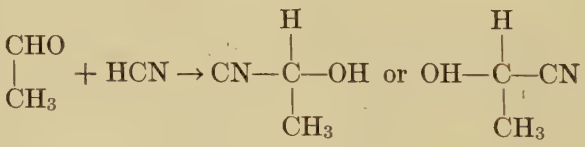
This is the only method available for regenerating a sugar from an osazone. The method is of historical interest, for Fischer established by its use the structure of synthetic α-acrose. It affords the means of preparing the corresponding *ketose* from an *aldose*.

$$\text{Aldose} \rightarrow \text{hydrazone} \rightarrow \text{osazone} \rightarrow \text{osone} \rightarrow \text{ketose}.$$

The asymmetric di-substituted hydrazines, such as methyl-phenylhydrazine, $C_6H_5—NCH_3—NH_2$, do not form osazones with glucose because they cannot act as oxidizing agents. Fructose, however, which already has a carbonyl group on the α-carbon is able to form a characteristic methyl-phenylosazone. Methyl-phenylhydrazine also forms a characteristic hydrazone with galactose, and diphenylhydrazine forms a characteristic hydrazone with arabinose.

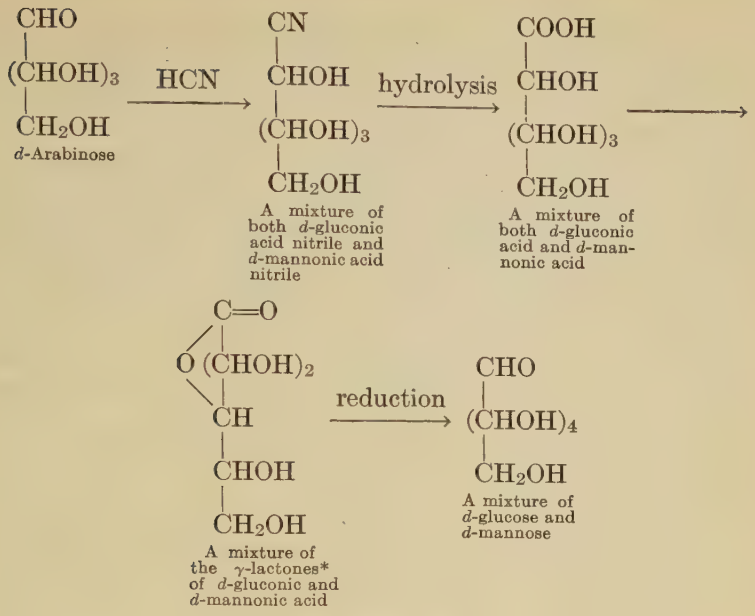
The Cyanhydrin Synthesis of Kiliani.—Both aldehydes and ketones react with HCN to form nitriles which, when hydrolyzed, give acids containing one carbon atom more than the original aldehyde or ketone. Consequently, this reaction can be utilized to go from a C_5 sugar to a C_6 sugar, etc. The lactones of the acids formed from carbohydrates by this reaction can be reduced with sodium amalgam to yield the corresponding aldose containing one more carbon atom than the original aldose.

Two stereoisomeric nitriles are usually formed at the same time, for we are introducing a new asymmetric carbon atom into the aldehyde formula of the sugar,



The synthesis should, and usually does, result in a racemic mixture of *d*- and *l*-forms; however, *mannose* and *fructose* form only a single nitrile. This is an *asymmetric synthesis*, resulting in an optically active product. Such syntheses are very rare in the organic laboratory. We do not know the explanation for any one of the few asymmetric syntheses which are on record.

By the method of Kiliani, we can advance from formaldehyde to a biose, and so on, one carbon at a time, to the higher sugars. Fischer carried glucose and mannose up to *aldo-nonoses* (C_9). *d*-Arabinose yields two hexoses, *d*-glucose and *d*-mannose, indicating that the only difference in the structural configuration of glucose and mannose must be in the α-carbon atom.



* Or more probably δ-lactones.

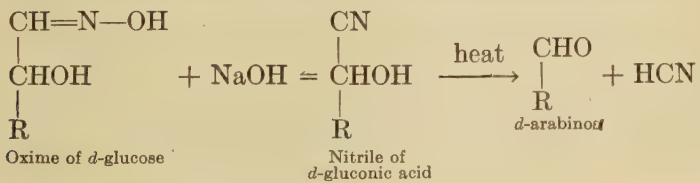
The acids may be separated by fractional crystallization before the *lactones* are reduced. An alternative view starts with the oxide form of the sugar (the more or less equilibrium mixture of α- and β-forms). The oxide ring breaks and adds HCN. The presence of α- and β-forms in unequal amounts explains why the corresponding nitriles are formed in unequal amounts. *d*-arabinose yields a preponderance of the levorotatory mannonic acid.

The Degradation of a Sugar.—(Preparing carbohydrates which contain one less carbon atom than the original sugar.)

1. In *Wohl's method* the aldoxime is prepared from the aldose by condensation with hydroxylamine,

$$R—CHO + NH_2OH = R—CH=N—OH + H_2O.$$

By treating the aldoxime of glucose, for example, with concentrated sodium hydroxide, it is converted into the nitrile of gluconic acid, and on heating this nitrile, HCN is eliminated and a pentose is formed.



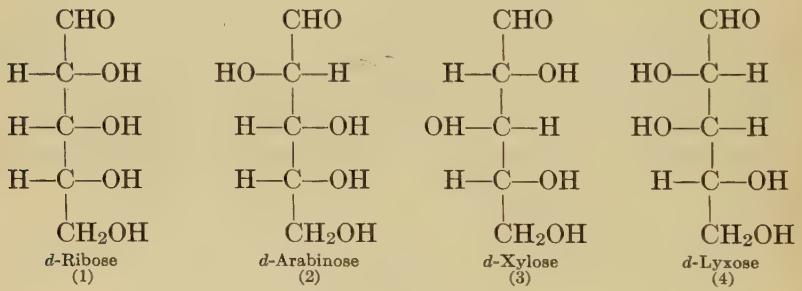
In practice it is preferable to heat the oxime with acetic anhydride and anhydrous zinc chloride. A vigorous reaction results, yielding the penta-acetyl derivative of gluconic acid nitrile, from which the —CN is eliminated by ammoniacal silver oxide.

2. In *Ruff's method* the aldose is oxidized to the corresponding acid, and the calcium salt of the acid is further treated with hydrogen peroxide in the presence of ferrous ions, resulting in the loss of carbon dioxide from the acid and the formation of a pentose. Neuberg electrolyzes the copper salt of the acid, using platinum electrodes, and obtains reduction to the pentose and carbon dioxide.

By the use of these methods it is possible to pass from glucose to formaldehyde, *one carbon being eliminated at a time.* Wohl's method is the one ordinarily used. In general, the yields are good.

Stereoisomerism.—Determination of Configuration of Pentoses and Hexoses[23] (cf. Armstrong[24] and Stewart[25]).

I. *The Pentoses.*—There are eight possible aldo pentoses, corresponding to the *d*- and *l*- forms of arabinose, xylose, ribose, and lyxose. There are thus four alternative forms for the *d*-modifications, which may be written as follows:



The *l*- forms are the mirror images of these.

A study of the various pentoses shows the following facts:

A. Arabinose and ribose form the same osazone. Therefore, their configuration must be identical except for the α-carbon atom. Only (1) and (2) or (3) and (4) answer this arrangement.

B. Arabinose on oxidation gives an *optically active* dibasic acid, ribose and xylose yielding optically inactive compounds. Sugars (2)

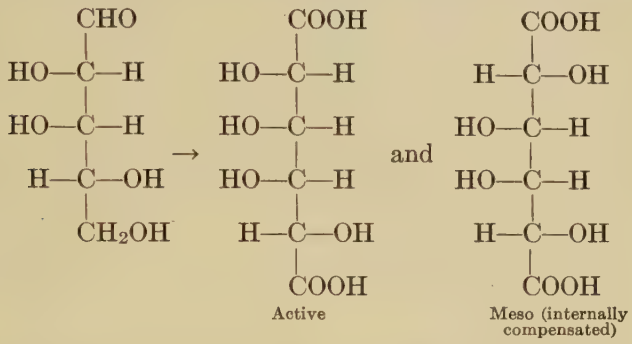
[23] The same methods which are used here are used to determine the structure of new compounds found in nature.

[24] Armstrong, E. F., The Carbohydrates and the Glucosides, Fourth Edition, pp. 33-56, Longmans, Green and Company, London (1924).

[25] Stewart, A. W., Stereochemistry, Longmans, Green and Company, London (1907).

and (4) will give optically *active* dibasic acids: (1) and (3) will yield optically *inactive* dibasic acids. Therefore, arabinose is either (2) or (4), ribose and xylose are (1) and (3), and lyxose is either (4) or (2).

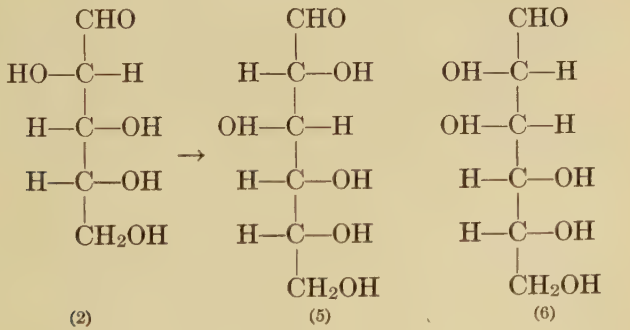
C. When HCN is added (Kiliani's reaction), a new asymmetric carbon atom is introduced into the molecule. The resulting hexose will therefore have two possible configurations. When the two hexoses from arabinose are oxidized, they give *optically active* acids, while those from lyxose give two acids, one active and one inactive. Number (4) is the only one which will form both active and inactive C_6 dibasic acids.



Therefore, lyxose is (4), arabinose (2), ribose (1), and by elimination xylose is (3).

II. *The Hexoses.*—From the formulae of the pentoses we obtain the formulae of the hexoses. A study of the various hexoses shows the following facts:

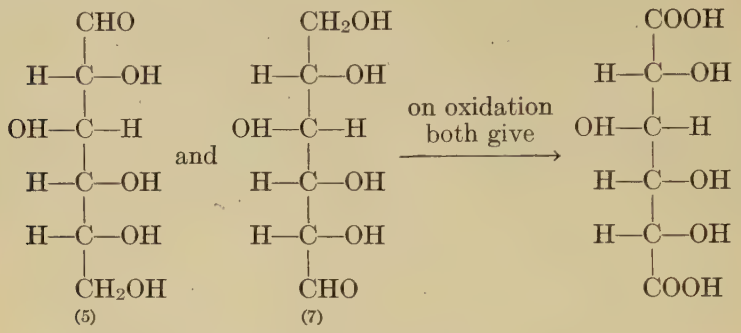
A. Arabinose treated with HCN yields two hexoses, glucose, and mannose.



Therefore, glucose must be either (5) or (6).

B. Both glucose and gulose give the same dibasic acid on oxidation, *i.e.*, saccharic acid. This means the 4 asymmetric carbon atoms are the

same. This can only occur when the terminal —CH_2OH and —CHO groups are interchanged as in (7).



In the case of (6) which is already symmetrical, it makes *no difference* in the transposition, *i.e.*, the same sugar results, no matter on which end, the —CHO and —CH_2OH groups occur. Therefore, glucose is (5), mannose is (6), and gulose is (7).

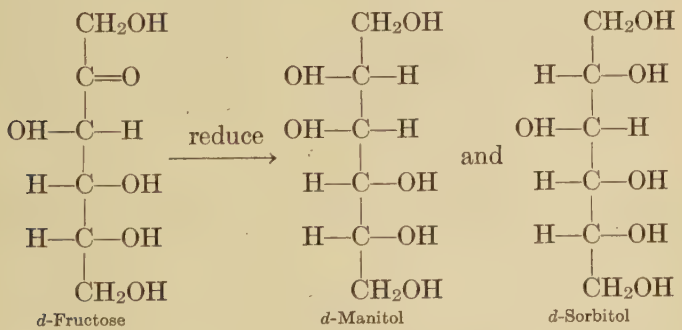
An extension of this reasoning gives the formulae of all the hexoses.

CHAPTER XXIII

CHEMICAL REACTIONS OF THE MONOSACCHARIDES

We have already discussed, in a consideration of the stereoisomerism of the pentoses and hexoses, the methods whereby monosaccharides are built up to sugars containing additional carbon atoms or whereby they are broken down to sugars containing a smaller number of carbon atoms. In this discussion it has been necessary to consider the reactions of the monosaccharides with phenylhydrazine, hydrocyanic acid, and hydroxylamine. In addition to these reactions there are a number of other reactions characteristic of the monosaccharides which will be considered in this chapter.

The Reduction of the Monosaccharides.—The reduction of a sugar gives rise to a hydroxy alcohol. Only one alcohol is formed by the reduction of an aldose. Two isomeric alcohols are formed by the reduction of a keto sugar. It is by the reduction of a keto sugar that we are able to determine the configuration of keto sugars, such as fructose. Fructose gives rise upon reduction to two alcohols, *d*-mannitol and *d*-sorbitol.



The space formula for *d*-mannitol can be easily obtained from the corresponding formula of *d*-mannose. Similarly the space formula of *d*-sorbitol can be obtained from the space formula of *d*-glucose. The two alcohols differ only in the configuration of the groups attached to the α-carbon atom. Therefore, the space configuration of the groups

attached to the remainder of the fructose molecule is proven. The same line of reasoning has been applied to the determination of the structure of the C_7 sugar, sedoheptose.[1, 2]

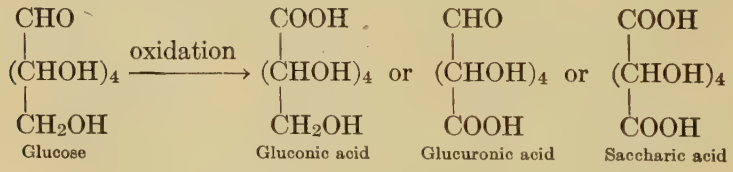
Both mannitol and sorbitol occur in plants. *d*-Mannitol is by far the most abundant of all the naturally-occurring hexahydroxy alcohols. It occurs in the onion, carrot, turnip, pineapple, and in the higher and lower fungi. In the fungi especially it may exceed glucose in amount or even displace it entirely. It is a normal constituent of silage, being formed by the bacterial reduction of fructose (from sucrose).

Mannitol is readily soluble in hot alcohol, sparingly soluble in cold alcohol, which fact allows for its ready isolation by direct crystallization. The amount present in silage ranges from 0.52 per cent to 2.54 per cent in average samples, although corn silage has been reported to contain as much as 3.3 per cent, and sunflower silage 5.61 per cent (cf. Dox and Plaisance[3, 4]).

Toward the close of the World War the suggestion was made that nitro mannitol be used as a high explosive, securing the mannitol from silage juices. *d*-Mannitol can easily be made by the catalytic hydrogenation of mannose.

d-Sorbitol occurs in the ripe fruits of the mountain ash and in most of the fruits of the *Rosaceae*. It can be readily prepared by hydrogenating *d*-glucose.

The Oxidation of the Sugars.—Glucose on oxidation yields three different acids, all containing six carbon atoms:



d-Glucose, when oxidized with bromine, yields *d*-gluconic acid. This acid is of interest, because when it is heated with pyridine or quinoline,

[1] La Forge, F. B., and Hudson, C. S., Sedoheptose, A New Sugar from *Sedum spectabile*. I., *J. Biol. Chem.*, 30 : 61–77 (1917).

[2] La Forge, F. B., Sedoheptose, A New Sugar from *Sedum spectabile*. II., *J. Biol. Chem.*, 42 : 367–374 (1920).

[3] Dox, A. W., and Plaisance, G. P., The Occurrence of Mannite in Silage and Its Possible Utilization in the Manufacture of Explosives, *Science*, 46 : 192–193 (1917).

[4] Dox, A. W., and Plaisance, G. P., The Occurrence and Significance of Mannitol in Silage, *J. Am. Chem. Soc.*, 39 : 2078–2087 (1917); also *Res. Bull.* No. 42, Iowa Agr. Exp. Sta., Dec., 1917.

it is partly converted into its stereoisomeric isomer, or antimere, *i.e.*, *d*-mannonic acid. The gluconic to mannonic acid reaction is important, because it affords a means of passing from glucose to mannose, and in a similar way one may pass from galactose to talose, and from xylose to lyxose. Apparently the —H and —OH groups on the carbon atoms adjacent to the carboxyl group interchange positions. Pyridine and quinoline prevent the formation of the lactone of the acids which, if present, would prevent the change from taking place.

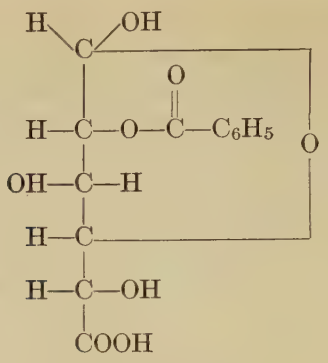
Spoehr has reported the presence of glucuronic acid as a plant constituent. Physiologically it is one of the most important of the oxidation products. It is apparently formed only when the aldehyde group is " protected " by being combined with some other compound in a glucoside-like linkage. It is frequently found in urine associated, in such a glucoside-like compound, with other substances. When certain substances, *e.g.*, camphor, are taken into the body and it is difficult for the body to oxidize them, they are in many instances combined in the body with glucose to form glucosides. One end of the glucose chain is thus protected from oxidation, but the other end oxidizes to a carboxyl group, so that a glucuronic acid compound results. These conjugated compounds are then excreted. This appears to be the way in which more or less injurious and difficultly oxidizable compounds are dealt with by both plants and animals, *e.g.*, the glucosides in plants which contain hydrocyanic acid, benzaldehyde, etc. It is apparently a means which the organism uses to protect itself against toxic agents. The plant glucosides may be compared in physiological importance to the animal glucuronic acid derivatives.

Griffith[5] notes that when sodium benzoate is administered to rabbits, a part of the benzoic acid is eliminated in combination with glycine, as hippuric acid, but that in spite of this fact a very appreciable amount is likewise excreted as benzoyl glucuronic acid, which in certain of his experiments amounted to one-third of the total combined benzoic acid which was excreted.

Quick[6] observed that, when benzoic acid is fed to a dog, it is excreted not as hippuric acid but as *β*-*d*-glucuronic-acid-*α*-monobenzoate. Quick further notes that the linkage between the glucuronic acid and the benzoic acid is not a glucosidal linkage, for the aldehyde group is still free. He accordingly suggests the following formula:

[5] Griffith, W. H., Benzoylated Amino Acids in the Animal Organism. III. A Method for the Determination of Hippuric Acid and a Study of the Synthesis of Hippuric Acid in Rabbits, *J. Biol. Chem.*, 69 : 197–208 (1926).

[6] Quick, A. J., The Preparation and Study of *β*-*d*-Glycuronic Acid Monobenzoate (Benzoyl Glycuronic Acid), *J. Biol. Chem.*, 69 : 549–563 (1926).



In the light of what we have already learned in regard to the recent revision of the structure of the oxide ring, the above formula should probably be a δ-oxide rather than a γ-oxide.

Saccharic acid is formed by the action of nitric acid on glucose. The sparingly soluble monopotassium salt serves as a test for glucose. *Mucic acid* is the corresponding acid of galactose. Thirty-five to forty per cent nitric acid at 85° will produce the best yields of mucic acid. It has a sandy, crystalline appearance and has taken on added importance within the last few years, inasmuch as it is being manufactured from the galactans of the wood of the western larch and may be used in various ways to replace the more expensive tartaric acid.

The *keto sugars* on oxidation break at the carbonyl group and give rise to two acids; fructose, for example, gives glycolic acid, $CH_2OH—COOH$, and trihydroxy butyric acid.

Dissociation and Molecular Rearrangements.—*The Action of Alkalies on Monosaccharides.*—Recent investigations on the chemical reactions of the sugars have thrown light on reactions and physiological processes in metabolism.

In vitro, pure solutions of sugars are stable. In the body they are extremely unstable and capable of numerous rearrangements and disintegrations. A study of the reactions of sugars to acids, bases, and salts may afford a clue to the reason for their behavior *in vivo*, including the mechanism of their decomposition and the nature of the products formed.

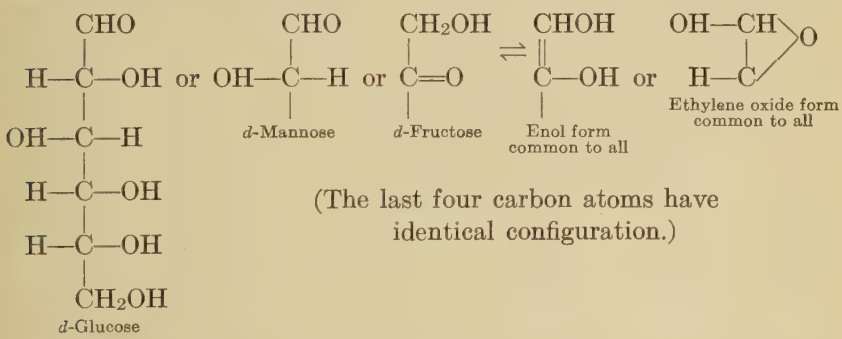
Sugars act as very weak acids. Michaelis and Rona[7] give the following dissociation constants at 18°: glucose, 6.6×10^{-13}; fructose, 9.0×10^{-13}; galactose, 5.2×10^{-13}; mannose, 10.9×10^{-13}; sucrose, 2.4×10^{-12}; maltose, 18.0×10^{-13}; lactose, 6.10×10^{-13}. That the

[7] Michaelis, L., and Rona, P., Die Dissoziationskonstanten einiger sehr schwacher Säuren, insbesondere der Kohlenhydrate, gemessen auf elektometrischem Wege, *Biochem. Z.*, 49 : 232–248 (1913).

sugars are *very* weak acids may be seen by comparison with the dissociation constants of some of the weak acids; acetic acid, 1.8×10^{-5}; butyric acid, 1.5×10^{-5}; lactic acid, 1.4×10^{-4}; boric acid (H^+ and H_2BO_4), 1.7×10^{-9}; and hydrocyanic acid, 4.7×10^{-10}. Ethyl alcohol forms sodium ethylate very easily; glycerol forms glycerolates with three sodium atoms. Sugars fall in the same class. Their importance lies in the fact that these metallic compounds decompose much more easily than does the original sugar.

Nef, over a period of more than ten years, studied the reactions of alkalies on sugars. He found that all the compounds which were formed were very unstable and that they decomposed into a large number of substances.

A. If the concentration of hydroxyl ions is very low, a molecular rearrangement of the sugar molecule results. This rearrangement is known as the Lobry de Bruyn[8-10] transformation. Thus, if *d*-glucose is treated with a solution of N/20 calcium hydroxide, the optical rotation changes with time at 15° to 20° to a new equilibrium. Starting with either *d*-glucose, *d*-fructose, or *d*-mannose, the same equilibrium is reached, and an equilibrium mixture is obtained containing *d*-glucose, *d*-fructose, *d*-mannose, α- and β-*d*-glutose, and *d*-pseudo fructose. These reciprocal relations are explained as being due to the conversion of the sugar into an enol form common to all.



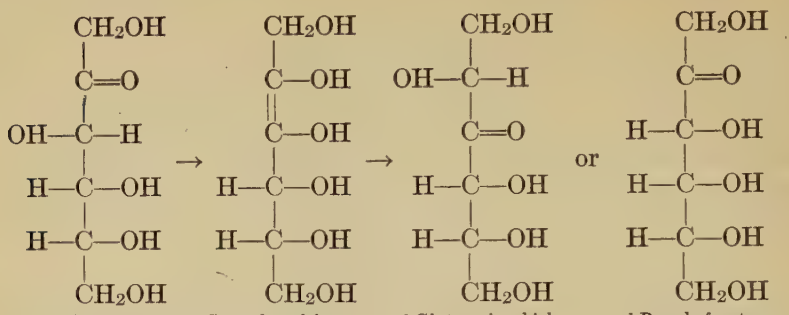
(The last four carbon atoms have identical configuration.)

From the enol form or the ethylene oxide form, it can be easily seen that either glucose, fructose, or mannose can be regenerated. It is also possible for fructose to yield a second enolic form:

[8] Lobry de Bruyn, C. A., Action des alcalis dilués sur les hydrates de carbone I, *Rec. trav. Chim.*, 14 : 156–165 (1895).

[9] Lobry de Bruyn, C. A., and Ekenstein, W. A. van, Action des alcalis sur les sucres. II. Transformation reciproque des uns dans les autres des sucres glucose, fructose et mannose, *Rec. trav. Chim.*, 14 : 203–216 (1895).

[10] Ekenstein, W. A. van, and Blanksma, J. J., Transformation du *l*-gulose et du *l*-idose en *l*-sorbose, *Rec. trav. Chim.*, 27 : 1–4 (1908).



Benedict, Dakin, and West[11] have studied the conditions of this transformation with particular reference to the formation of glutose. They find that either glucose or fructose can be readily partially converted into glutose by treatment with solutions of calcium hydroxide at 75° to 80° in the absence of air. (Two kilos of invert sugar dissolved in 8 liters of water, to which the $Ca(OH)_2$ derived from 30 grams of CaO had been added; the flask was then evacuated and heated three to four hours at 75° to 80°. The fermentable sugars were then removed by addition of 300 grams of starch-free yeast and the residual glutose syrup concentrated and purified.) They note that the diabetic organism does not utilize glutose and that the tolerance of man and animals for glutose is extremely low. In most instances, the greater part is excreted unchanged. Insulin shock is not affected by the injection of glutose. It does not form a hexose phosphate; neither is it fermentable by yeast.

Spoehr and Wilbur[12] note that the Lobry de Bruyn transformation takes place in disodium phosphate solutions, the aldo sugars being partially converted into ketoses and vice versa. *d*-Glutose was formed from *d*-glucose in good yield, and they give explicit directions for the preparation of *d*-glutose. They note that apparently the Lobry de Bruyn transformation does not represent a true equilibrium, for in their experiments *d*-glutose was not transformed back into glucose and fructose when it alone was treated with disodium phosphate solutions. Spoehr even goes so far as to raise the question as to whether we actually know the true formula for *d*-glutose.

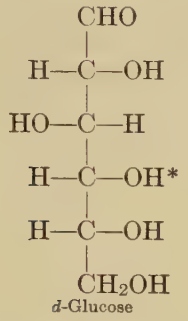
In a similar manner the *d*-galactose series yields an equilibrium mix-

[11] Benedict, E. M., Dakin, H. D., and West, R., On Glutose and Its Biochemical Behavior, *J. Biol. Chem.*, 68 : 1–14 (1926).

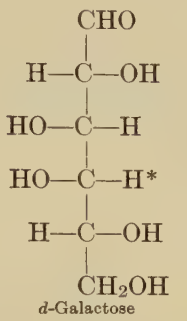
[12] Spoehr, H. A., and Wilbur, P. C., The Effect of Disodium Phosphate on *d*-Glucose and *d*-Fructose, *J. Biol. Chem.*, 69 : 421–434 (1926).

ture containing *d*-galactose, *d*-talose, *d*-tagatose, *d*-sorbose, and α- and β-*d*-galtose.

The sugars of the *glucose* series are never converted into those of the *galactose* series, because the difference between the two series lies in the configuration of the γ-carbon atom.



d-Glucose



d-Galactose

Apparently the rearrangements involve only the α- and β-carbon atoms, the molecule being most reactive at the carbonyl end.

It is interesting to compare the ratio of the amounts of sugars found in nature and in this equilibrium mixture. *d*-Glucose and *d*-fructose are the most important of the naturally-occurring hexoses and are found in greatest abundance. *d*-Mannose, *d*-galactose, and *l*-sorbose are the only others found at all commonly in nature.

Nef found ketoses and aldoses in about equal proportion in his equilibrium mixture. In the glucose series, *d*-glucose and *d*-mannose were the only aldoses present, and they were in a ratio of 5 glucose : 1 mannose. In the galactose series, *d*-galactose accounted for 90 per cent of the aldoses which were present. Accordingly, the nature and proportion of the sugars present in the equilibrium mixture closely approach the nature and proportion of the sugars occurring in plant products.

Wolfrom and Lewis,[13] in a study of the alkali-carbohydrate equilibrium, used extremely mild methods for the production of the equilibrium mixture. Under such conditions, they find that *d*-glucose was converted by the dilute alkali into a mixture of 63.4 per cent *d*-glucose, 30.9 per cent *d*-fructose, and 2.4 per cent *d*-mannose, leaving a balance of only 3.3 per cent which they state is composed of "non-sugar substances, probably saccharinic acids." Wolfrom and Lewis then studied the equilibrium mixture obtained from tetramethyl glucose in the presence of dilute alkali. In this instance, no keto sugars were formed, the equilibrium mixture containing an equal amount of tetramethyl glucose

[13] Wolfrom, M. L., and Lewis, W. L., The Reactivity of the Methylated Sugars. II. The Action of Dilute Alkali on Tetramethyl Glucose, *J. Am. Chem. Soc.*, 50 : 837–854 (1928).

and tetramethyl mannose. Figure 115 shows the interconversion of tetramethyl mannose and tetramethyl glucose into the equilibrium mixture consisting of 50 per cent tetramethyl mannose and 50 per cent tetramethyl glucose. Wolfrom and Lewis note that the same equilibrium is reached from both directions and that the rate of conversion of tetramethyl glucose is the same as the rate of the conversion of the tetramethyl mannose. They suggest that in tetramethyl glucose only the 1.2 di-enol can form and that the enol formation is not due to hydration followed by a loss of water, but is actually due to the shifting of a hydrogen atom.

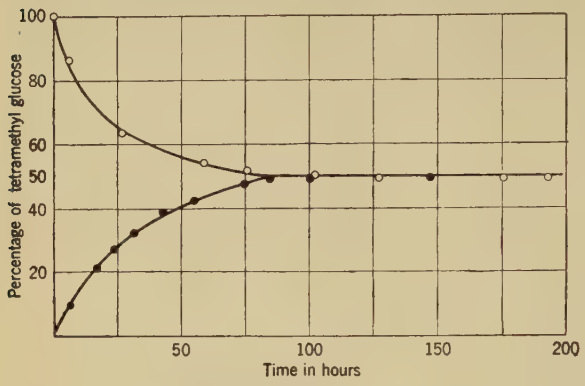
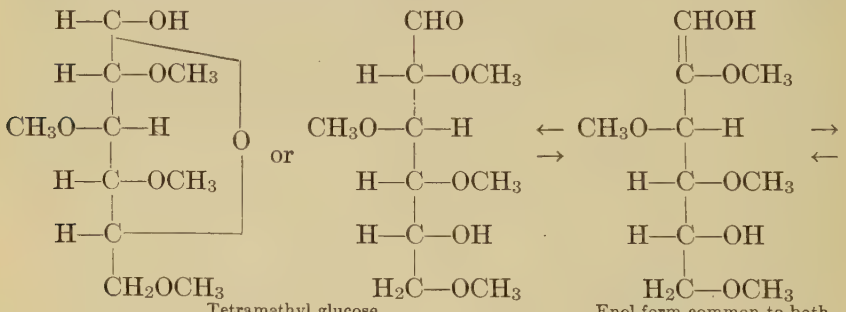
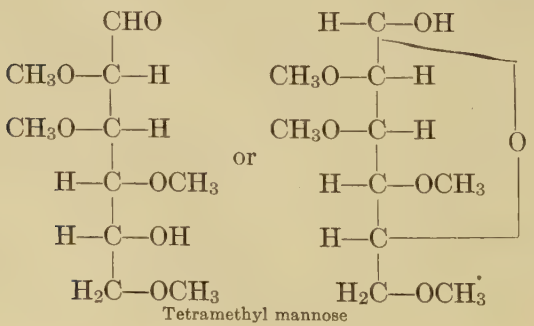


FIG. 115.—Showing the interconversion of tetramethyl mannose (solid dots) and tetramethyl glucose (open circles). (Data of Wolfrom and Lewis)





B. In the higher concentrations of alkali, oxidation results. Mathews[14] placed 2 grams of sugar in a 400 cc. flask and added 50 cc. of 0.4 N KOH, shaking the mixture constantly, and measured by means of changes in pressure, the oxygen which was absorbed by the solution. He found that all sugars oxidized spontaneously in the presence of air. Glucose, galactose, maltose, and lactose oxidized at approximately the same rate but less rapidly than did fructose. In certain experiments he allowed sugars to stand for a time in contact with alkali in an oxygen-free atmosphere, *e.g.*, the air replaced by hydrogen. When oxygen was admitted to such solutions, it was rapidly absorbed, indicating that the

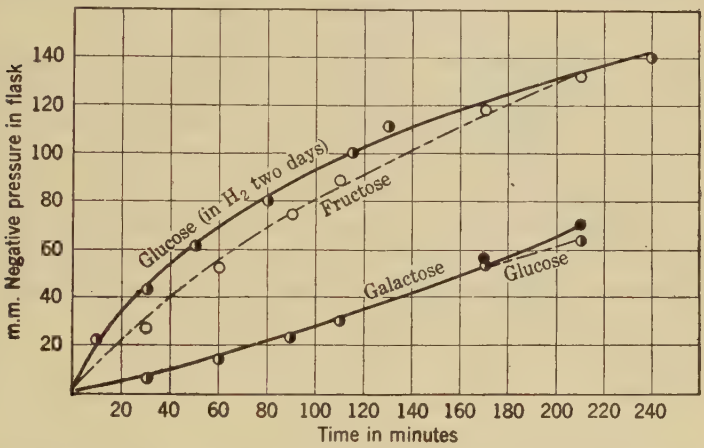


FIG. 116.—Showing the rate of absorption of oxygen by solutions of glucose, fructose, and galactose dissolved in 0.4 N KOH solutions. Also the increased reactivity of a glucose-NaOH solution after standing for two days in a hydrogen atmosphere. (Data of Mathews.)

rearrangement produced by the alkali changes the rate with which sugars combine with oxygen. The theory for this change in rate assumes that the glucose molecule has been broken up into actively reducing fragments. Figure 116, taken from Mathews' data, shows the rate of absorption of oxygen for glucose, fructose, and mannose, and for galactose, in the presence of alkali. It also shows the curve where glucose was treated with alkali in the absence of oxygen, and oxygen was later admitted to the system.

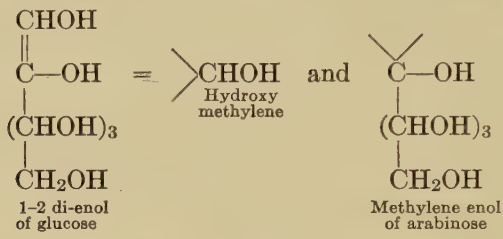
Nef,[15] as a result of his studies, presented a theory for the oxidation

[14] Mathews, A. P., Spontaneous Oxidation of the Sugars, *J. Biol. Chem.*, 6 : 3–20 (1909).

[15] Nef, J. U., Dissociationsvorgänge in der Zuckergruppe, *Ann.*, 357 : 214–312 (1907); *Ann.*, 403 : 204–383 (1914).

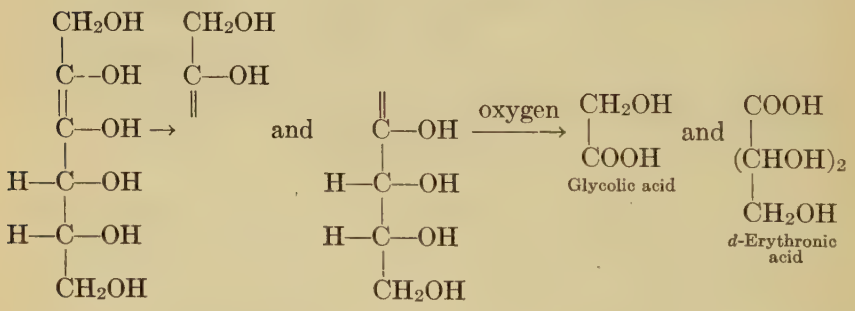
of the sugars in alkaline solution. He assumed that in the presence of alkali the hexoses form 1–2, 2–3, and 3–4 di-enols, and that beside giving a mixture of six sugars, these di-enols split at the double linkage into a mixture of various carbohydrate-like compounds containing 1, 2, 3, 4, and 5 carbon atoms, the rupture at the double bond yielding radicals which contain *divalent carbon.*

Thus, the 1–2 di-enol of glucose would give rise to hydroxy methylene (divalent carbon) and the methylene enol of arabinose (again divalent



carbon). Inasmuch as divalent carbon should be an exceedingly reactive form, these radicals would absorb oxygen to yield formic acid from the hydroxy-methylene, and *d*-arabonic acid (with some *d*-ribonic acid) from the methylene enol of arabinose.

The 2–3 di-enol gives the methylene enol of a diose and of *d*-erythrose,



which on exposure to oxygen will yield glycolic acid and erythronic acid (some *l*-threonic acid is also formed). *d*-Erythronic acid is the chief optically active trihydroxy butyric acid found among the oxidation products of *d*-glucose when air or hydrogen peroxide is used as oxidizing agent in the presence of six equivalents of sodium hydroxide.

The 3–4 di-enols break up into two molecules of the methylene enol of glyceric aldehyde, $CH_2OH—CHOH—COH$, which oxidize to *d*- and *l*-glyceric acid.

Under ordinary conditions (alkali only) none of the six sugars present in the equilibrium mixture oxidize to C_6 acids. This, however, takes place when Fehling's solution is used (cf. Glattfeld[16]).

The factors which must be controlled in an exact study of the reactions of sugars in alkaline solutions are (at least):

1. The concentration of alkali (or salt);
2. The oxidation potential (*i.e.*, air, H_2O_2, $KMNO_4$, $Cu(OH)_2$, AgO, etc.);
3. Temperature;
4. Time.

The earlier work in this field was carried out without exact control of hydrogen ion concentration and without exact measurements of oxidation potential by the more modern technic. Evans[17] and his students have been repeating and extending the observations of Nef, using more exact physico-chemical control of the systems, and have shown the importance of all of the four items noted above, as governing the nature of the reaction.

The different sugars show a wide variation in the proportion of the products which are formed. Carbon dioxide, formic acid, and oxalic acid may be obtained. Alkaline silver oxide is perhaps the most drastic of all the oxidizing agents, for by its use the sugars yield almost exclusively carbonic, formic, and oxalic acids.

While the sugars are very weak acids and poorly ionized, their salts are highly ionized. This increases the concentration of the sugar ions in solution, and it is thought that the greater reactivity is due to these unstable sugar ions. The carbon atoms decrease in activity as their distance from the carbonyl group increases.

Nef has shown that glucose in the presence of sodium hydroxide reacts to yield an equilibrium mixture containing at least 93 different compounds, and concluded that,

1. The initial fragments undergo molecular rearrangement to form more stable compounds.
2. They may react with each other, one being oxidized and the other reduced.

[16] Glattfeld, J. W. E., On the Oxidation of *d*-Glucose in Alkaline Solution by Air as well as by Hydrogen Peroxide, *Am. Chem. J.*, 50 : 135–157 (1913).

[17] Evans, W. L., et al., The Mechanism of Carbohydrate Oxidation, I–IX, *J. Am. Chem. Soc.*, 47 : 3085–3098, 3098–3101, 3102–3105 (1925), 48 : 2665–2677, 2678–2681, 2703–2714 (1926); 50 : 486–492, 1496–1503, 2267–2285 (1928).

3. They may combine or polymerize.
4. They may react with other substances, such as oxygen, to form acids, etc.

If we include synthetic steps, glucose and sodium hydroxide will yield, according to theory, no less than 116 compounds, but positive evidence has been obtained for only 93.

Because of this ease of dissociation and decomposition with the liberation of energy, the sugars can be used as energy sources by living organisms, both under anaerobic and aerobic conditions. In vital processes, *e.g.*, in the cell, the sugar is not in a pure aqueous solution but in a colloidal system in the presence of considerable amounts of inorganic salts and reactive chemical groups. Such conditions lead to a dissociation of the sugar molecnle, and the ions in turn break up, liberating energy. Spoehr believes that enzymes are simply special means for bringing about this dissociation. If the enzymes formed salts with the sugars which later dissociated, we would have the reactive sugar ions set free to decompose. Undoubtedly rearrangement and decomposition of the molecule precede metabolic utilization of the sugars.

Spoehr and Smith[18, 19] have studied the oxidation of glucose in the presence of oxygen and various catalysts. They note that sodium ferropyrophosphate acts as a catalyst for the oxidation of carbohydrates, so that they are easily oxidized by atmospheric oxygen. The work of Spoehr has been commented upon and extended by the observations of Palit and Dhar,[20] who report the oxidation of carbohydrates in an essentially neutral solution in the presence of cerous hydroxide or ferrous hydroxide as catalysts. These authors believe that the oxidation of carbohydrates in the cell is catalyzed by certain of the cell constituents, so as to bring about the formation of the reactive sugar radicals.

Power and Upson[21] oxidized *d*-glucose by drawing air through a somewhat alkaline solution. Using 100 grams of *d*-glucose dissolved in 10 liters of saturated calcium hydroxide, they found that 95 grams of the glucose was oxidized by drawing air through the solution for 236 hours. They obtained 4.87 grams of carbon dioxide, 23.1 grams of volatile acids,

[18] Spoehr, H. A., and Smith, J. H. C., Studies on Atmospheric Oxidation. I. The Oxidation of Glucose and Related Substances in the Presence of Sodium Ferro-Pyrophosphate, *J. Am. Chem. Soc.*, 48 : 236–248 (1926).

[19] Smith, J. H. C., and Spoehr, H. A., Studies on Atmospheric Oxidation. II. The Kinetics of the Oxidation with Sodium Ferro-Pyrophosphate, *J. Am. Chem. Soc.*, 48 : 107–112 (1926).

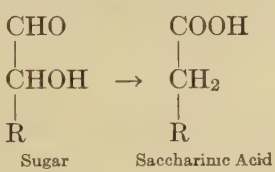
[20] Palit, C. C., and Dhar, N. R., Catalytic and Induced Oxidation of Some Carbohydrates, Uric Acid, and Inorganic Substances, *J. Phys. Chem.*, 30 : 939–953 (1926).

[21] Power, M. H., and Upson, F. W., The Oxidation of *d*-Glucose by Air in Calcium Hydroxide Solution, *J. Am. Chem. Soc.*, 48 : 195–202 (1926).

mainly formic acid, and 84.9 grams of non-volatile acids, in which mixture they identified *d*-arabonic acid, *d*-erythronic acid, *l*-glyceric acid, glycolic acid, and oxalic acid (4.1 grams). Fructose under similar conditions gave a somewhat lower yield of carbon dioxide (4.28 grams) and somewhat more oxalic aid (9.1 grams).

Witzemann[22] has studied the oxidation of glucose with hydrogen peroxide in the presence of disodium phosphate and states that disodium phosphate is the only chemical substance that is known to be generally necessary to the life of organisms which is able to catalyze the quantitative oxidation of glucose to carbon dioxide and water. Under laboratory conditions he was able to bring about the complete oxidation of glucose with hydrogen peroxide in the presence of disodium phosphate. We know that hydrogen peroxide is a product of cell activity and that phosphates are present in all cells, so that possibly the reactions which he studied in the laboratory are analogous to reactions taking place in living organisms. In a later paper, Witzemann[23] studied the oxidation of sugars in the presence of guanidine as a catalyst and finds that the reactions in the presence of this organic base are very similar to the reactions in the presence of sodium and potassium hydroxides.

Saccharinic acids are formed by internal oxidation and reduction of aldo-monosaccharides, and possess the same empirical formula as the



Sugar Saccharinic Acid

sugar from which they are derived. They are ordinarily formed in alkaline solution, in the absence of an oxidizing agent (cf. Nef[24]).

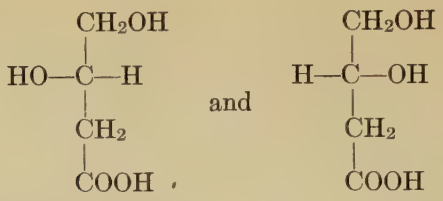
Glattfeld and Miller[25] isolated a C_4-saccharinic acid from oxidation products of maltose in the presence of alkali and air. This is the first instance where the alkaline oxidation of a sugar yielded a saccharinic acid. The acid which was isolated was *d-l*-2–3-dioxy butyric acid.

[22] Witzemann, E. J., Disodium Phosphate as a Catalyst for the Quantitative Oxidation of Glucose to Carbon Dioxide with Hydrogen Peroxide, *J. Biol. Chem.*, 45 : 1–22 (1920).

[23] Witzemann, E. J., The Action of Guanidine upon Glucose in the Presence and Absence of Oxygen, *J. Am. Chem. Soc.*, 46 : 790–794 (1924).

[24] Nef, J. U., Dissoziationsvorgänge in der Zuckergruppe, *Ann.* 376 : 1–119 (1910).

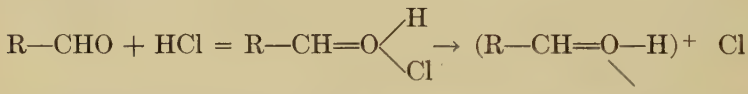
[25] Glattfeld, J. W. E., and Miller, G. E., The C_4–Saccharinic Acids. I. The Resolution of *dl*-2, 3-Dioxybutyric Acid into the Optically-Active Components. The Derivatives of These Acids, *J. Am. Chem. Soc.*, 42 : 2314–2321 (1920).



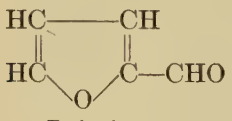
l-2-3-Dioxy butyric acid *d*-2-3-Dioxy butyric acid

The Action of Acids on Carbohydrates.—The sugar molecule is the most stable in neutral solution and in the absence of metallic salts. It is the least stable in the form of a salt. The decomposition reactions usually proceed at a slower rate in acid solution than under alkaline conditions, and some of the primary decomposition products formed in acid solutions can be readily isolated.

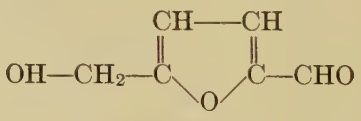
Apparently the first reaction is that of a salt formation, the formation of oxonium salts on the carbonyl group (tetravalent oxygen) and subsequent ionization.



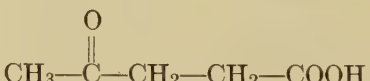
Rearrangement then takes place with a loss of water and the formation of furfural from pentoses and of levulinic acid and formic acid or of hydroxy-methyl furfural from hexoses.



Furfural



Hydroxy-methyl furfural



Levulinic acid

Other products may be formed, depending upon the experimental conditions.

The formation of furfural from pentoses is of importance from the standpoint of laboratory technic, inasmuch as this reaction is utilized for the quantitative determination of pentoses and pentose-containing materials. Numerous methods have been devised for the quantitative production of the theoretical amount of furfural from the pentose material and for the subsequent quantitative estimation of the furfural which was formed. The usual method employed is to boil the carbohydrate-

containing material with 12 per cent hydrochloric acid, collecting the furfural in the distillate and determining the furfural either by precipitating it as the relatively insoluble furfural-phloroglucide or by estimating the furfural directly by either some titration method or by an appropriate colorimetric method. Pervier and Gortner[26] studied the conditions necessary for the quantitative production of furfural from pentose material and outlined conditions whereby the theoretical amount of furfural may be obtained. They likewise described a new titrametric method for the estimation of the furfural.

Recently, Suminokura and Nakahara[27] have stated that xylidine gives a characteristic color reaction with furfural but not with methyl furfural or hydroxy-methyl furfural, and they note a colorimetric procedure for the estimation of furfural in the presence of small amounts of hydroxy-methyl furfural or methyl furfural. Inasmuch as hexoses form a small amount of hydroxy-methyl furfural and rhamnose yields methyl furfural, the older methods for the estimation of the true pentoses have always yielded somewhat uncertain results, due to the possible contamination of the furfural, which was formed, with these other similar compounds. If further work bears out the contention of Suminokura and Nakahara, their method will probably replace all of the present methods for the estimation of furfural.

The Laboratory Synthesis of Monosaccharides.—The synthesis of *d*-glucose by Emil Fischer was one of the outstanding achievements of the organic chemist.

1. As already noted the first synthesis of a sugar was that by Butlerow in 1861. He treated formaldehyde with saturated calcium hydroxide solution and secured a sweet syrup having the properties of a carbohydrate. This sugar, Loew named "formose."

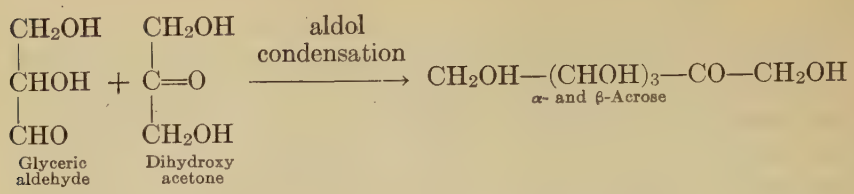
2. Fischer and Tafel (1887–1889) treated acrolein dibromide with barium hydroxide and secured a sweet syrup containing two products which they called "α- and β-acrose."

$$CH_2Br—CHBr—CHO + Ba(OH)_2 = C_6H_{12}O_6 + 2BaBr_2.$$

3. Fischer later used glycerose as the starting point for a sugar synthesis. Crude glycerose is a mixture of glyceric aldehyde and dihydroxy acetone. These apparently undergo aldol condensation with the formation of a ketose which has the same formula as fructose:

[26] Pervier, N. C., and Gortner, R. A., The Estimation of Pentoses and Pentosans, I. The Formation and Distillation of Furfural, II. The Determination of Furfural, *Ind. Eng. Chem.*, 15 : 1167–1169, 1255–1262 (1923).

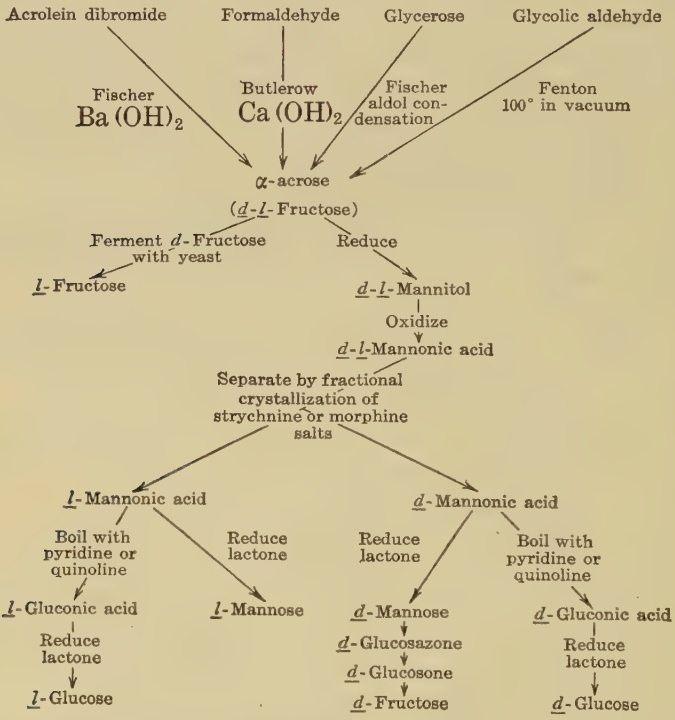
[27] Suminokura, K., and Nakahara, Z., A New Colorimetric Microdetermination of Furfural, *Trans. Tottori Soc. Agr. Sci.* (Japan), 1 : 148–159 (1928).



This was again a mixture of α- and β-acrose and its osazone differed from that of glucose only by being optically inactive.

4. Fenton,[28] in 1897, heated glycolic aldehyde in a vacuum at 100° and secured a solid transparent "glass." This again contained α-acrose.

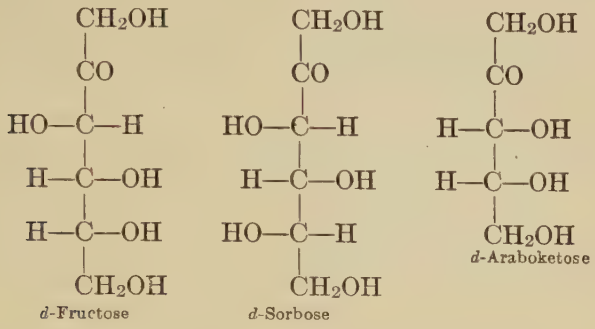
From his studies Fischer concluded that α-acrose was *d*-*l*-fructose, and he succeeded in proving this and in synthesizing from the synthetic sugar, *l*-fructose, *d*-fructose, *l*-mannose, *d*-mannose, *l*-glucose, and *d*-glucose. His scheme of synthesis was as follows:



Thus the structure of α-acrose was proven. It was not until 1924, however, that we had any knowledge as to the nature of β-acrose.

[28] Fenton, H. J. H., A New Synthesis in the Sugar Group, *J. Chem. Soc.*, London, 71 : 375–383 (1897).

Küster and Schoder[29] have now shown β-acrose to be *d-l*-sorbose and have also shown that there is present a considerable amount of a keto pentose which they call " araboketose." The space formulae for these sugars are:



The work of Baly on the synthesis of sugars has already been mentioned. Irvine and Francis report that in their study of Baly's synthetic sugar, they find no ketoses. Therefore, Baly's synthetic sugar is not α- and β-acrose. The crude syrup contained about 26.5 per cent of *aldoses*. While glucose was not definitely proven, it is at least suggestive that the condensation of " active formaldehyde " by ultraviolet light to form a sugar does not proceed in the same direction as the polymerization of formaldehyde by chemical means.

The Conversion of Glucose to Galactose.—No one has as yet been able to convert glucose into galactose *in vitro*, but *in vivo* this conversion apparently takes place readily in the mammary gland where lactose is formed. Glucose injected into the blood of a lactating animal causes lactosuria; when the mammary gland is not active, glucosuria results. It is for this reason that we believe the seat of transformation is in the mammary gland and that it is probably of enzymatic nature, although no enzyme has ever been isolated which will cause the transformation.

Fermentation of Pentoses and Hexoses.—This phase of carbohydrate study is extremely interesting because by this reaction energy is released and valuable industrial products are formed. It likewise involves problems of chemical configuration and biological specificity.[30]

[29] Küster, W., and Schoder, F., Über das Entstehen von Sorbose bei der Kondensation des Formaldehyds, *Z. physiol. Chem.*, 141 : 110–131 (1924).

[30] For those who may be interested in a more extended discussion of the intermediate products of yeast fermentation, cf. Nord, F. F., Chemical Processes in Fermentations, *Chem. Rev.*, 3 : 41–79 (1926).

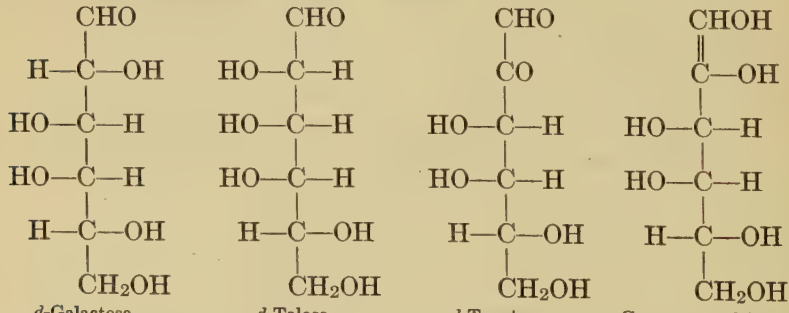
The transformations are caused by yeasts, bacteria, moulds, etc., and are, of course, in the last analysis dependent upon enzymes.

Pasteur treated *d*-*l*-tartaric acid with *Penicillium glaucum* and found that the *d*-form was utilized by the organism, leaving the *l*-form unattacked. If ammonium lactate were present, the *l*-form only was utilized. *Penicillium glaucum* uses only the *l*-form of mandelic acid (phenyl glycolic acid, C_6H_5—CHOH—COOH), whereas the yeast, *Saccharomyces ellipsoideus*) uses only the *d*-form.

Yeast ferments only *d*-glucose, *d*-mannose, *d*-fructose, and *d*-galactose. These four *naturally-occurring* sugars are the only ones which are fermented. The synthetic *l*-forms remain unaltered. *d*-Glucose, *d*-fructose, and *d*-mannose are fermented with the same ease, and the rate of fermentation of these three sugars has the same temperature coefficient. This is explained by the fact that they have a similar space configuration. It has already been pointed out that they are inter-convertible and that they have the same enolic form.

It may be assumed that one of the necessary steps in fermentation is enolization. The enol forms, then breaking down to ethyl alcohol and carbon dioxide, the break being at the double bond of the enol formula. Further proof that enolization is probably the deciding factor lies in the fact that the α- and β-methyl glucosides and gluconic acids are not fermentable. Their terminal carbon atom differs from the configuration of the terminal carbon of glucose, and they cannot enolize.

Galactose ferments with difficulty; some yeasts do not ferment it at all. The fermentation curve, with temperature as a variable, is decidedly different from the curve for glucose, fructose, and mannose. The fermentation of galactose, therefore, is probably a different reaction. Possibly a different enzyme is involved in the enolization of galactose. Rather interestingly, however, talose and tagatose which have the same enol form as galactose are not fermentable by any known yeast.

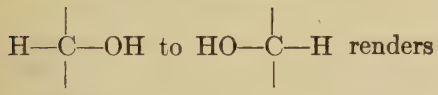


It is interesting to note that in talose the upper two carbon atoms

have the same configuration as in mannose and the lower four the same configuration as in galactose; nevertheless talose is not fermentable, again a striking example of biological specificity.

There is a very close relationship between structure and the enzymes which cause fermentation. None of the ordinary yeasts ferment the pentoses. Apparently only the three-carbon sugars are readily fermentable *i.e.*, trioses, hexoses, and nonoses. (The available data state that the one known nonose is fermentable. This point needs to be verified).

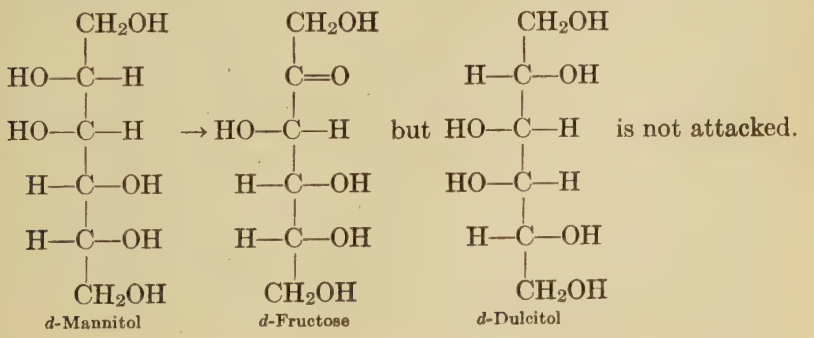
The same specificity has already been noted in the case of the glucosides when the β-glucosides are hydrolyzed by emulsin and the α-glucosides by maltase. However, the α-methyl-*d*-xyloside, which is identical with α-methyl-*d*-glucoside except for dropping one carbon atom, is not hydrolyzed by these enzymes. Shortening the chain by one carbon atom spoils the harmony of enzyme action. Galactosides are not hydrolyzed by these enzymes; *l*-glucosides are not hydrolyzed by any enzyme; *d*-mannosides are not hydrolyzed (a single shifting from



H—C—OH to HO—C—H renders them immune to attack).

Fischer has likened the close relationship between enzyme and substrate to a lock and key. Only a specific key will open a specific lock! However, changing an —OH group to an —OR group does not spoil the harmony. Lactose is hydrolyzed by lactase which also hydrolyzes β-galactosides. Therefore, we know that lactose is a glucose-β-galactoside.

The Action of Microorganisms on Sugars.—*Bacterium xylinum* oxidizes aldoses to monobasic acids and oxidizes certain alcohols to ketoses, but apparently oxidizes only such alcohols as have a =CHOH group adjacent to a $-CH_2OH$ group and when the —OH of the adjacent =CHOH group is of a configuration similar to the =CHOH group being oxidized. For example,



Within the last few years we have seen a large chemical industry develop, making use of biological organisms in order to produce desirable organic compounds from carbohydrates. The organisms used include yeasts, bacteria, and the fungi. Of course, certain of these reactions have been used commercially for many years, such as alcohol by yeast fermentation, vinegar (acetic acid) by the acetic bacteria, and lactic acid by the lactic acid bacillus. We have now, however, controlled fermentations which will produce acetone, *n*-butyl alcohol, amyl alcohol, various aliphatic aldehydes, formic acid, citric acid, etc. In many instances, pentoses may be utilized, including the carbohydrates in such waste products as corn cobs (cf. Fred and Peterson, et al.[31-36], and Gabriel[37]).

This particular field of study offers attractive possibilities for the development of commercial sources of many organic compounds.

Certain of the fungi are not specific in their fermentation requirements. Anderson,[38] Letcher and Willaman,[39] and White and Willaman[40] have shown that the flax wilt organism, *Fusarium lini*, will ferment to ethyl alcohol and carbon dioxide almost any naturally-

[31] Peterson, W. H., and Fred, E. B., The Role of Pentose-Fermenting Bacteria in the Production of Corn Silage, *J. Biol. Chem.*, 41 : 181–186 (1920); Fermentation of Fructose by Lactobacillus Pentoaceticus, N. Sp., *J. Biol. Chem.*, 41 : 431–450 (1920); The Fermentation of Glucose, Galactose, and Mannose by Lactobacillus Pentoaceticus, N. Sp., *J. Biol. Chem.*, 42 : 273–287 (1920); The Production of Acetaldehyde by Certain Pentose-Fermenting Bacteria, *J. Biol. Chem.*, 44 : 29–46 (1920).

[32] Fred, E. B., Peterson, W. H., and Davenport, A., Fermentation Characteristics of Certain Pentose-Destroying Bacteria, *J. Biol. Chem.*, 42 : 175–189 (1920).

[33] Fred, E. B., Peterson, W. H., and Anderson, J. A., The Relation of Lactic Acid Bacteria to Corn Silage, *J. Biol. Chem.*, 46 : 319–327 (1921).

[34] Peterson, W. H., Fred, E. B., and Verhulst, J. H., The Destruction of Pentosans in the Formation of Silage, *J. Biol. Chem.*, 46 : 329–338 (1921).

[35] Fred, E. B., and Peterson, W. H., Fermentation Process for the Production of Acetic and Lactic Acids from Corncobs, *Ind. Eng. Chem.*, 13 : 211–213 (1921).

[36] Peterson, W. H., Fred, E. B., and Verhulst, J. H., A Fermentation Process for the Production of Acetone, Alcohol, and Volatile Acids from Corncobs, *Ind. Eng. Chem.*, 13 : 757–759 (1921).

[37] Gabriel, C. L., Butanol Fermentation Process, *Ind. Eng. Chem.*, 20 : 1063–1067 (1928).

[38] Anderson, A. K., Biochemistry of Plant Diseases, The Biochemistry of *Fusarium lini* Bolley, Minnesota Studies in Plant Science, Studies in the Biological Sciences, No. 5, pp. 237–280 (1924).

[39] Letcher, H., and Willaman, J. J., Biochemistry of Plant Diseases. VIII. Alcoholic Fermentation of *Fusarium lini*, *Phytopathology*, 16 : 941–949 (1926).

[40] White, Mollie G., and Willaman, J. J., Biochemistry of Plant Diseases. X. Fermentation of Pentoses by *Fusarium lini*, *Biochem. J.*, 22 : 583–591 (1928); Biochemistry of Plant Diseases. XI. *Fusarium lini* and the Pyruvic Acid Theory of Alcoholic Fermentation, *Biochem. J.*, 22 : 592–595 (1928).

occurring sugar, either pentose or hexose, with equal ease, and when the carbohydrate is exhausted, will utilize the ethyl alcohol, oxidizing it to carbon dioxide and water.

Pentose-fermenting organisms are widely distributed in nature. The lactic and acetic fermentations of sauerkraut and silage are largely due to their activity.[41] Dox and Yoder[42] state that in silage the pentoses, sucrose, glucose, and fructose, are readily fermented but that the starch is not attacked and remains unaltered in the silage.

Bacteria and fungi in particular are capable in many instances of utilizing pentoses as their sole source of carbon, while other organisms may be wholly unable to utilize them. One of the organisms which does not attack pentoses is bakers' yeast, although it readily ferments the hexoses. Bakers' yeast may therefore be added to the mixture of hexoses and pentoses, and the hexoses fermented off, and then the pentoses remaining may be determined by Fehling's solution in the usual manner. If we know the original total reduction, the difference in the two determinations gives the hexose sugars which were fermented (cf. Davis and Sawyer[43]). Spoehr has used this method to follow the fate of carbohydrates in wheat seedlings, germinated and grown in the dark, and he finds that the pentoses decrease with the increase in age of the seedling.

Only recently some of the rare sugars have come into use for the identification of bacteria. Kendall[44-47] and his coworkers state that certain rare sugars are as specific for certain bacteria as are chemical reagents for certain organic compounds. Kendall has published extensively on the subject of using certain bacteria to identify traces of a sugar which is the only one they will attack.

The Transformation of the Various Groups of Sugars in the Plant.—The transformation of the sugars into other groups within the plant appears to be independent of the primary process of photo-

[41] Note the papers by Fred, Peterson, et al., already cited.

[42] Dox, A. W., and Yoder, L., Influence of Fermentation on the Starch Content of Experimental Silage, *J. Agr. Res.*, 19 : 173–179 (1920).

[43] Davis, W. A., and Sawyer, G. C., The Estimation of Carbohydrates. IV. The Presence of Free Pentoses in Plant Extracts and the Influence of Other Sugars on Their Estimation, *J. Agr. Sci.*, 6 : 406–412 (1914).

[44] Kendall, A. I., Bacteria as Chemical Reagents, *Chem. Met. Eng.*, 24 : 56–60 (1921).

[45] Kendall, A. I., et al., Studies in Bacterial Metabolism, LXVI–LXIX, *J. Infectious Diseases*, 32 : 355–383 (1923).

[46] Kendall, A. I., Bacterial Metabolism, *Physiol. Rev.*, 3 : 438–455 (1923).

[47] Kendall, A. I., Bacteria and the Chemist, *Ind. Eng. Chem.*, 15 : 1001–1002 (1923).

synthesis. Spoehr,[48] in his studies on the cacti found that the carbohydrate ratios were controlled by at least two factors, *water content and temperature.* In his experiments with low water content and high temperature, he found an increase in polysaccharides and pentosans and a decrease in monosaccharides.

It may well be that the increase in pentosans was a response of the plants toward the elaboration of hydrophilic colloids to bind the water in the plant against the forces of transpiration and to build up an imbibition pressure to draw water from an already water-depleted soil. We have already noted the work of Newton, showing that native grasses of Alberta " bind " water in the semi-arid areas as the season advances.

Spoehr found that a high water content and a low temperature reversed the conditions, causing an increase in monosaccharides and a decrease in polysaccharides and pentosans. It is to be regretted that similar experiments were not conducted for the other possible series, *i.e.*, high water—high temperature and low water—low temperature.

Apparently a fairly complex equilibrium is involved, probably controlled by enzyme action, and bound water content and temperature affect inversion and reversion of carbohydrates. Other workers have shown that at low temperatures the synthesis of starch from monosaccharides is interfered with and takes place only when the glucose content of the tissue fluids reaches a relatively high level. It has been observed, for example, that the needles of conifers accumulate starch during the actively growing season but that at the onset of winter temperatures the starch disappears, being converted into soluble sugars, chiefly glucose, so that in the northern portion of the United States the needles become quite starch-free early in the winter period. With the advent of the warmer days in the spring the starch reappears and the glucose content decreases.

The question of the relationship between temperature and carbohydrate transformation may become of economic importance. For example, in the storage of potatoes, there appear to be at least three reactions involved:

1. The starch → sugar transformation;
2. The sugar → starch transformation;
3. The sugar → carbon dioxide and water transformation (the respiration process).

The rate of all these reactions is decreased by a lowering of the temperature, but the last two are decreased relatively more than is the

[48] Spoehr, H. A., The Carbohydrate Economy of Cacti, Carnegie Institution of Washington, Publication No. 287 (1919).

first one, so that below a certain point the tubers become "sweet." The critical temperature appears to lie somewhere between +4.5 and +6.0° C. If the storage temperature is lower than +4.5, soluble sugars increase, and at 0° C. approximately 3 per cent of sugar accumulates before equilibrium is reached.

Russow[49] was apparently the first person to describe the disappearance of starch in plant tissues under the influence of low temperatures. He noted that starch was present at the onset of winter in relatively large quantities in the woody stems of various plants, but that during the three winter months, December, January, and February, the starch had

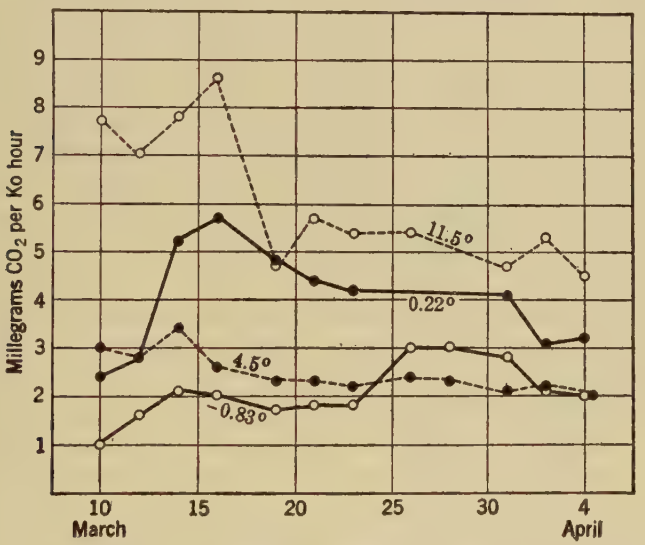


FIG. 117.—The respiration of tubers of russet rural potatoes at −0.83°, +0.22°, +4.5°, and +11.5° C., plotted against time. (Data of Hopkins.)

almost completely disappeared, being replaced by oils or fats. In controlled experiments he was able to demonstrate a decrease in starch content when the woody tissue was placed under cold conditions, and a reappearance of the starch when such twigs were later raised to a higher temperature.

Müller-Thurgau,[50] Appleman,[51,52] Butler,[53] Bartholomew,[54] Hop-

[49] Russow, *Naturforscher-Gesellschaft bei der Universitaet Dorpat*, Jan., 1882, [cf. translation by Hamilton P. Traub, Dr. Russow on the Disappearance and Reappearance of Starch in the Bark of Woody Plants, *The Minnesota Horticulturist*, 55 : 241–242 (1927)].

[50] Müller-Thurgau, H., Über Zuckeranhäufung in Pflanzentheilen in Folge niederer Temperatur, *Landw. Jahrb.*, 11 : 751–828 (1882).

kins,[55] and others have noted and studied these changes. Perhaps the most recent study is that of Hopkins. He found that there was a marked acceleration in the rate of respiration of potato tubers at 0° C., such that for a very considerable period the rate of respiration was greater at this temperature than at +4.5°. This stimulation passed through a maximum and then decreased, but even after many days the rate of respiration was still greater than was that at +4.5°. Certain of his data are shown in Fig. 117.

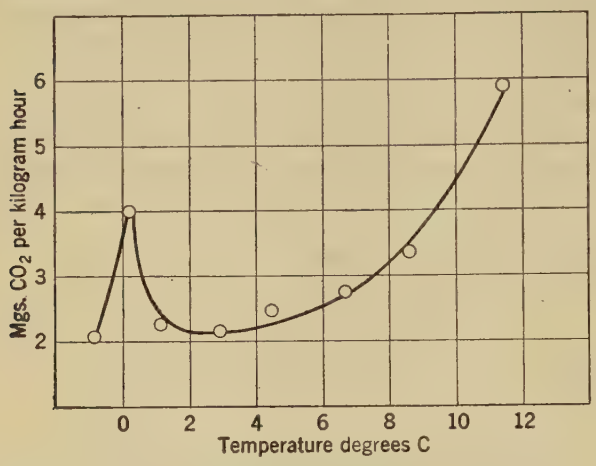


FIG. 118.—The respiration of potato tubers at various temperatures. (Data of Hopkins.)

In a study of the rate of respiration with temperature, over the range from −0.83° to +11.5°, he noted a minimum point in the respiration curve at about +3.0°, as shown in Fig. 118.

In a study of the carbohydrate changes at various temperatures, Hopkins found that the sugar accumulation at 0° C. is at first slow, then rapidly increases, and finally slowly decreases. At +4.5°, the sugar content is nearly constant. At +1.17°, it increases fairly rapidly from the start, while at −0.83°, there is a gradual increase in total sugars but a decrease in reducing sugars. These data are shown in Fig. 119.

Hopkins suggests that the acceleration of respiration at 0° C. is dependent upon an increased concentration of sugar and that up to a certain concentration, sugar increases respiration, and beyond that point inhibits it.

[51] Appleman, C. O., Changes in Irish Potatoes During Storage, Bull. 167, Maryland Agr. Exp. Sta. (25th Ann. Report) pp. 327–334 (1912).

[52] Appleman, C. O., Biochemical and Physiological Study of the Rest Period in the Tubers of *Solanum tuberosum*, *Bot. Gaz.*, 61 : 265–294 (1916).

[53] Butler, O., The Significance of Sugar in the Tubers of *Solanum tuberosum*, *Bull. Torrey Bot. Club*, 40 : 110–118 (1913).

[54] Bartholomew, E. T., A Pathological and Physiological Study of the Black Heart of Potato Tubers, *Centr. Bakt., Parasitenk.*, 43 : 609–639 (1915).

[55] Hopkins, E. F., Relation of Low Temperatures to Respiration and Carbohydrate Changes in Potato Tubers, *Bot. Gaz.*, 78 : 311–325 (1924).

Wolff[56] has studied the sugar ⇌ starch equilibrium in the potato over a somewhat wider range of temperatures than was covered by the studies of Hopkins. Certain of Wolff's data are shown in Fig. 120. Wolff notes that the equilibrium is shifted not only by temperature changes but also by changes in the water content, and suggests that the area of sugar formation shown for the higher temperatures is in reality governed by changes in water relationships. Rather interestingly he notes that the pulp from macerated potatoes does not show an increased sugar content upon drying, such as is shown by the intact tuber, indicating rather definitely that gel structure or tuber structure influences the equilibrium.

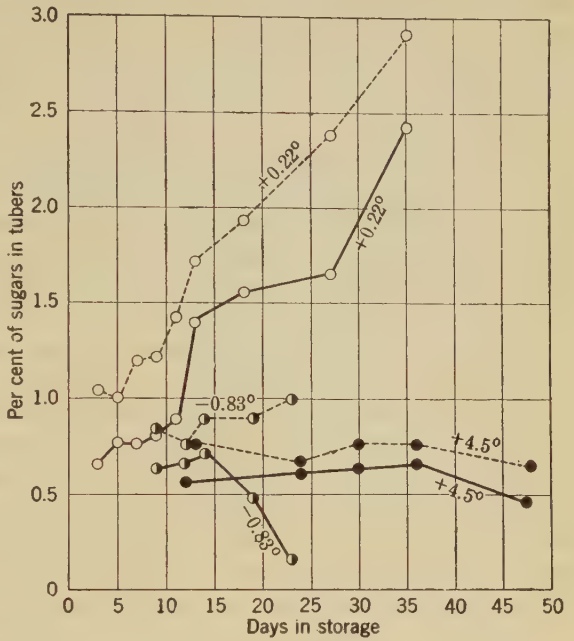


FIG. 119. — Showing carbohydrate changes in potato tubers held at low temperatures. Reducing sugars shown by solid lines, total sugars by broken lines. (Data of Hopkins.)

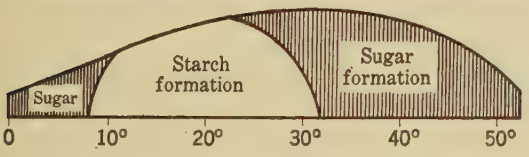


FIG. 120.—Showing diagrammatically the temperature effect on the sugar ⇌ starch equilibrium in the potato. (After de Wolff.)

Hasselbring and Hawkins,[57] and Hasselbring[58] have made a study of sweet potato tubers and found that during growth the tubers contain very small

[56] Wolff, C. J. de, Die Saccharosebildung in Kartoffeln während des Trocknens. I., *Biochem. Z.*, 176 : 225–245 (1926).

[57] Hasselbring, H., and Hawkins, L. A., Physiological Changes in Sweet Potatoes During Storage, *J. Agr. Res.*, 3 : 331–342 (1915).

[58] Hasselbring, H., Behavior of Sweet Potatoes in the Ground, *J. Agr. Res.*, 12 : 9–17 (1918).

amounts of sugar, so that during the summer sweet potato tubers are "dry and tasteless." Following harvest, the starch → sugar transformation proceeds rapidly, first with the formation of reducing sugars and then of sucrose. The rate of starch decrease and sugar increase follows closely Van't Hoff's rule of the relation of temperature to chemical reactions, *i.e.*, the rate approximately doubles for a ten-degree increase in temperature. It is highly probable that the transformation of starch to sugar in the sweet potato is not primarily dependent upon a temperature reaction, inasmuch as Gore[59] notes that sweet potatoes, when ripe, contain a very powerful diastase, sufficient to hydrolyze all of the starch to sugars. As a matter of fact, Gore utilized this reaction to prepare a syrup from sweet potatoes.

Coville[60] has suggested that the influence of cold as a stimulant to plant growth may well be associated with the transformation of starch to sugar. He notes that many plants will not resume growth in continuously warm weather but will do so if subjected to a period of cold. In most of our trees and shrubs the reserve carbohydrate is starch stored during a period of warm or hot weather. At the beginning of autumn the sap wood is gorged with this material, and as the process of chilling goes on, this reserve carbohydrate is transformed more and more into soluble sugars. As spring approaches, the starch practically disappears. Coville found that in the dormant blueberry wood, taken in the spring, the ratio of sugar to starch was about seven times what it was in a similar tissue in the autumn. Possibly a similar phenomenon is responsible for the "sugar flow" in the hard maple.

Coville also advances a theory to explain this paradoxical stimulating effect of cold upon plants. He suggests that the starch grains stored in the plant cells are at first separated by living, active cell membranes, from the enzymes which hydrolyze the starch, but that when the cell is chilled, the membranes undergo a change in permeability, so that the enzyme "leaks" out and hydrolysis results. If Colville's explanation is correct, anaesthetics should have the same effect as low temperature.

The Origin of the Pentose Sugars.—The pentose sugars occur in plants mainly in the form of polysaccharides,—pentosans, for example in the form of gums such as xylan or araban. Free pentose sugars have, however, been demonstrated in plants by Davis and Sawyer.[61]

[59] Gore, H. C., Occurrence of Disastase in the Sweet Potato in Relation to the Preparation of Sweet Potato Syrup, *J. Biol. Chem.*, 44 : 19–20 (1920).

[60] Coville, F. V., The Influence of Cold in Stimulating the Growth of Plants, *J. Agr. Res.*, 20 : 151–160 (1920).

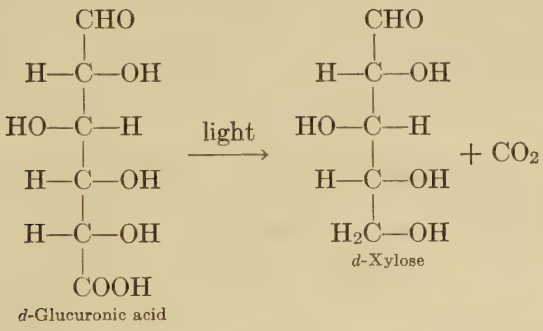
[61] Davis, W. A., and Sawyer, G. C., The Estimation of Carbohydrates. IV. The Presence of Free Pentoses in Plant Extracts and the Influence of Other Sugars on Their Estimation, *J. Agr. Sci.*, 6 : 406–412 (1914).

Pentose sugars are apparently of great physiological importance as evidenced by the fact that they are constant constituents of the nucleic acids. The question as to the origin and mode of formation of the pentoses resolves itself into the question as to whether they are primary products of photosynthesis or whether they are derived from other sugars by metabolic processes.

In the aldose sugars the first carbon atom (the —CHO group) is the reactive point and the reactions are mainly due to the carbonyl group. In the compound sugars and in the glucosides this group is combined in the molecule, so that it is taken out of the sphere of chemical reactivity. Under such conditions any chemical changes which occurred would take place on the $—CH_2OH$ group at the other end of the carbon chain. Such an oxidation would result in the formation of an aldehyde acid, such as glucuronic acid.

Spoehr found glucuronic acid to be present in cacti. This was an important observation, for it affords a rational theory for the formation of pentosans in plants. When a solution of glucuronic acid is exposed to sunlight, carbon dioxide is split off. This is a characteristic of many hydroxy acids. Malic acid ($COOH—CH_2—CH(OH)—COOH$), for example, loses two molecules of carbon dioxide and forms ethyl alcohol.

The natural pentoses belong to both the *l*-series and the *d*-series of sugars. *d*-Glucuronic acid on the loss of carbon dioxide forms *d*-xylose.



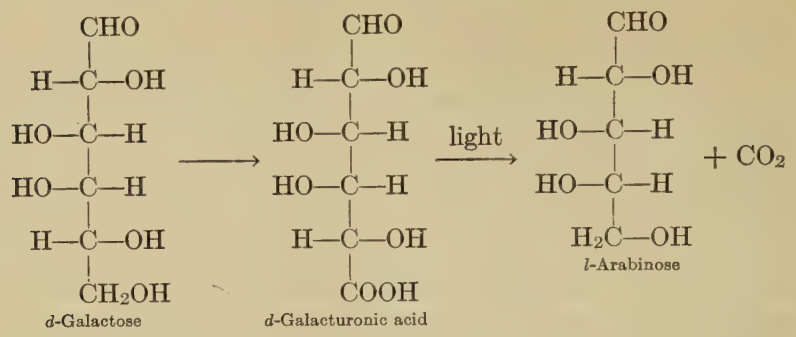
d-Glucuronic acid

d-Xylose

Direct oxidation of the aldehyde group and loss of carbon dioxide would have formed *d*-arabinose. It is a striking fact that *d*-glucose is usually associated in nature with *d*-xylose.

Similarly *d*-galactose forms *d*-galacturonic acid which loses carbon dioxide to form *l*-arabinose.

Here again the *d*-galactose is associated in nature with *l*-arabinose and not with *d*-lyxose, as would be the case if the aldehyde group were oxidized and carbon dioxide were lost.



Bacteria in certain instances are also able to reduce *d*-glucuronic acid to *d*-xylose.

The Significance of Pentosans in Plants.—Pentosans are the principal constituents of plant gums and mucilages. They are highly hydrophilic (German, "Pflanzenschleim") and undoubtedly play a major role in the water relations of certain plants, especially the succulent desert plants, such as the cacti. This highly hydrophilic property is not a property of the hexose polysaccharides. The relation of hydrophilic colloids in plants to water-retaining capacity has already been discussed under colloids.

Occurrence of Monosaccharides in Nature.—Glucose and fructose are the only two common monosaccharides to occur in the free state. Sorbose, a keto-hexose, and La Forge's two keto-heptoses (*d*-mannoketo heptose and sedoheptose) occur rarely. All of the other naturally-occurring sugars are found in some combined form, either as compound sugars, polysaccharides, glucosides, or in the form of their corresponding alcohols.

Mannose occurs as mannosans, anhydride-like condensation products which may be hydrolyzed to mannose by acids. The best source for the preparation of mannose is vegetable ivory, the endosperm of the seed of the tagua palm, *Phytelephas macrocarpa*, cf. Hudson and Sawyer.[62] Mannose is the true aldehyde of the alcohol, mannitol, and may be obtained by oxidizing the alcohol. Fischer first prepared it in this manner and later found that it occurred in nature.

Galactose occurs as a constituent of milk sugar (glucose-galactose) and of raffinose (galactose-glucose-fructose) and as the polymer, " galactan," in gums, mucilages, and pectin. It is usually associated in galactans with arabinose or xylose. Its occurrence as a galactoside is rare, except in milk sugar, and in the " saponins " where it is encountered

[62] Hudson, C. S., and Sawyer, H. L., The Preparation of Pure Crystalline Mannose and a Study of Its Mutarotation, *J. Am. Chem. Soc.*, 39 : 470–478 (1917).

rather frequently. It may be conveniently prepared from the wood of the western larch (cf. Schorger and Smith[63]).

Fructose, the sweetest of all the sugars, occurs free in most fruit juices. Honey is largely " invert sugar " and owes its superior sweetness to the fructose content. Its polysaccharide is inulin, the reserve carbohydrate in the tubers of the dahlia, chicory, and the Jerusalem artichoke. Various " inulides " also occur, usually associated with the true inulin.

The specific rotatory power of a fructose solution changes markedly with temperature, becoming *less negative* as the temperature increases. At 87.3° C. it becomes equal to and opposite to the optical rotation of glucose. Therefore, at 87.3° C. invert sugar has a zero rotation.

Fructose can be separated from solution as the calcium salt. The relative sweetening power of the sugars, on the basis of sucrose = 100, is shown in Table LVIII (cf. Biester, Wood, and Wahlin[64]). As a commercial sugar, fructose offers great possibilities because of its great sweetness. When the chemical engineering problems involved in its isolation on a large scale are solved, it will undoubtedly become a very important commercial product.

TABLE LVIII

The Relative Sweetness of the Various Sugars
(Data of Biester, Wood, and Wahlin)

Sucrose	100.0	Maltose	32.5
Fructose	173.3	Rhamnose	32.5
Invert sugar	130.0	Galactose	32.1
Glucose	74.3	Raffinose	22.6
Xylose	40.0	Lactose	16.0

[63] Schorger, A. W., and Smith, D. F., The Galactan of *Larix occidentalis*, *J. Ind. Eng. Chem.*, 8 : 494–499 (1916).

[64] Biester, Alice, Wood, M. W., and Wahlin, C. S., Carbohydrate Studies I. The Relative Sweetness of Pure Sugars, *Am. J. Physiol.*, 73 : 387–396 (1925).

CHAPTER XXIV

THE HIGHER SUGARS

As already noted under classification, the higher sugars may be divided into the disaccharides, trisaccharides, tetrasaccharides, etc., formed by the condensation of two, three, four, or more molecules of monosaccharides with the elimination of one molecule of water less than the total number of monosaccharide molecules which are involved.

The determination of the structure of a compound sugar is extremely difficult, as may be appreciated from the fact that there has been until very recently uncertainty regarding the actual structure of sucrose. The determination of structure involves:

1. The nature of the constituent sugars;
2. The stereochemical forms (α or β);
3. The location of the alcohol groups involved in the linkage;
4. The position of the internal oxygen rings in the compound sugar.

The method which has been used to study the structure has been to introduce into the molecule non-hydrolyzable residues (*e.g.*, methylate or acetylate all of the alcohol groups), and then hydrolyze the sugar into its component monosaccharide derivatives. From a study of the compounds thus obtained, in comparison with the synthetic products formed from the monosaccharides in other studies, it may be possible to locate the carbon atoms through which the linkage of the sugars takes place.

Buston and Schryver[1] isolated from cabbage leaves a new carbohydrate, having the formula, $C_3H_8O_4$, which is possibly a new type of disaccharide. From 90 kilos of cabbage leaves, 10.7 grams of the compound was obtained. It melts at 148°, is faintly sweet, is soluble in water, but insoluble in alcohol and other organic solvents. It forms a tribenzoyl derivative, does not form an osazone, does not reduce Fehling's solution and is not hydrolyzed by acids. Its formula is apparently

[1] Buston, H. W., and Schryver, S. B., The Isolation from Cabbage Leaves of a Carbohydrate, Hitherto Undescribed, Containing Three Carbon Atoms, *Biochem. J.*, 17 : 470–472 (1923).

$CH_2OH—CHOH—O—CH_2OH$, and it may be looked upon as a disaccharide formed from glycolic aldehyde and formaldehyde, *i.e.*, a diose and a monose uniting to form a triose of an unusual type. Its interest to us is only from the theoretical standpoint, as a possible indication of a minor side reaction taking place in nature.

Sugars of the Formula, $C_{12}H_{22}O_{11}$.—These are analogous to simple glucosides which on hydrolysis yield two molecules of hexose sugars, *e.g.*, sucrose yields glucose and fructose.

Both mineral and organic acids hydrolyze disaccharides to the corresponding monosaccharides. The hydrogen ion concentration is the important factor, and the hydrogen ion concentration may be relatively low, as compared with many hydrolytic reactions, and still cause rapid hydrolysis.

This hydrolysis of disaccharides by acids was one of the early methods which was employed to measure the hydrogen ion concentration of a solution. Likewise it was the first chemical reaction to be measured by physico-chemical means, *i.e.*, by the change in optical rotation of the solution as hydrolysis progressed.

The disaccharides differ widely in the ease of hydrolysis by acids. Sucrose is rapidly hydrolyzed at 20° by normal sulfuric acid, while lactose needs prolonged heating at 80° to accomplish the same degree of hydrolysis. Armstrong states that the rates of hydrolysis are sucrose 1240; maltose 1.27; lactose 1.00. Each of the naturally-occurring disaccharides is hydrolyzed by its own specific enzyme, *e.g.*, sucrase, maltase, lactase, etc. The enzyme hydrolysis is usually more rapid and more complete than is acid hydrolysis. There has recently been placed on the market a concentrated preparation of sucrase (invertase) to be used in preparing " invert syrup " from sucrose.

The disaccharides may be classified into two groups:

I. The reducing disaccharides have one or more of the aldehyde groups potentially functional.

II. The non-reducing disaccharides have no potentially functional aldehyde group.

I. Reducing Disaccharides

Maltose	α Glucose α glucoside
Lactose	β Glucose β galactoside
Isolactose	Glucose ? galactoside
Melibiose	β Glucose α galactoside
Turanose	Glucose ? fructoside
Gentiobiose or isomaltose	β Glucose β glucoside
Cellose or cellobiose	β Glucose β glucoside

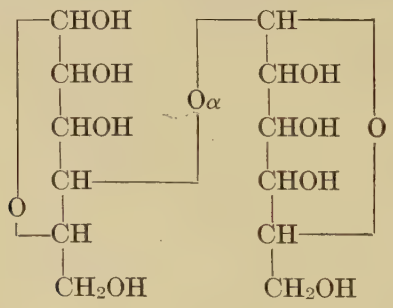
II. Non-Reducing Disaccharides

Sucrose	α-Glucose-α-(γ)-fructoside
Trehalose	α Glucose α glucoside
Isotrehalose	β Glucose β glucoside

The Reducing Disaccharides.—The only disaccharides of this group which are of sufficient importance for our consideration are maltose and lactose.

Maltose is the end product of the action of diastatic enzymes on starch. It was first isolated by De Saussure in 1819 from the products of the hydrolysis of starch. It reduces Fehling's solution, forms an osazone, is not hydrolyzed by sucrase, lactase, or emulsin, but is hydrolyzed by maltase to two molecules of glucose. Accordingly it can be regarded as a glucose α-glucoside. Maltose has eight free hydroxyl groups, inasmuch as it forms an octa-acetate.

The structure of maltose has been studied recently by Irvine and Black[2], by Cooper, Haworth, and Peat,[3] and by Haworth and Peat,[4] and as a result of their studies the structure of maltose is shown to be:



Cellobiose differs from maltose only in a stereochemical degree in that the oxygen linkage between the glucose radicals is β for cellobiose and α for maltose, cf. Zemplén.[5]

Pictet and Vogel[6, 7] report the synthesis of maltose by heating an

[2] Irvine, J. C., and Black, I. M. A., The Constitution of Maltose, *J. Chem. Soc.*, London, April, 1926, 862–875.

[3] Cooper, C. J. A., Haworth, W. N., and Peat, S., The Constitution of the Disaccharides. Pt. X. Maltose, *J. Chem. Soc.*, London, April, 1926, 876–880.

[4] Haworth, W. N., and Peat, S., The Constitution of the Disaccharides. Pt. XI. Maltose, *J. Chem. Soc.*, London, Dec., 1926, 3094–3101.

[5] Zemplén, G., Abbau der reduzierenden Biosen, I. Direkte Konstitutions-Ermittlung der Cellobiose, *Ber.* 59 : 1254–1266 (1926).

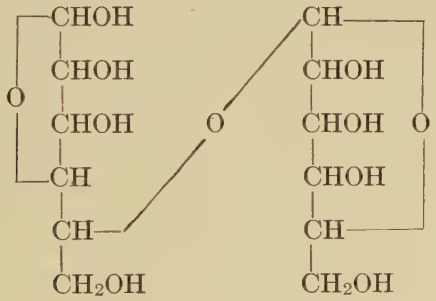
[6] Pictet, A., and Vogel, H., Synthèse du maltose, *Compt. rend.*, 184 : 1512–1514 (1927).

[7] Pictet, A., and Vogel, H., Synthèse du maltose, *Helv. Chim. Acta*, 10 : 588–593 (1927).

equimolecular mixture of α- and β-glucose in a vacuum at 160°. The mixture melts and then suddenly resolidifies with no change in temperature. The product which is formed appears to be a maltose hydrate, $C_{12}H_{22}O_{11} \cdot H_2O$, as shown by optical rotation, the melting point of the osazone, the acetate, and the nitrate, and by the optical rotation of the acetate and nitrate.

When starch is hydrolyzed by acid for the formation of glucose syrup or when *d*-glucose is acted upon by strong hydrochloric acid, a disaccharide isomeric with maltose is formed. This has been named " isomaltose " and has been considered as distinct from the other reducing disaccharides. Berlin[8] has shown that isomaltose is identical with gentiobiose, and notes that in the acid hydrolysis of starch, 5.7 per cent of the starch is converted into gentiobiose. Gentiobiose is non-fermentable by yeast, and in a later paper[9] Berlin gives directions for the preparation of gentiobiose from the non-fermentable residues.

Irvine and Black, and Cooper, Haworth, and Peat, in the papers already noted, give alternative formulae for maltose and gentiobiose. On the basis of the structural formula for maltose, already given, the structural formula for gentiobiose becomes:



Lactose (glucose β galactose) or milk sugar is the characteristic disaccharide of milk and is manufactured from milk whey. It reduces Fehling's solution, undergoes mutarotation, and forms an osazone. The potential aldehyde group belongs to the glucose radical, because upon oxidation and subsequent hydrolysis it yields galactose and gluconic acid.

Galactose occurs in the brain and in the medullary sheaths of the nerves as an important constituent of the glucolipides (*vide infra*). As

[8] Berlin, H., The Identity of Isomaltose with Gentiobiose, *J. Am. Chem. Soc.*, 48 : 1107–1111 (1926).

[9] Berlin, H., The Occurrence of Gentiobiose in the Products of the Commercial Hydrolysis of Corn Starch, *J. Am. Chem. Soc.*, 48 : 2627–2630 (1926).

already noted, we have no definite proof that any organ of the body, excepting the mammary gland, can transform glucose into galactose. It therefore appears that nature has made a provision for an adequate supply of galactose in the form of milk sugar during the early stages of brain development, and this consideration alone is sufficient to cast doubt upon the advisability of substituting sucrose for lactose in infant feeding.

Lactose is hydrolyzed by the specific enzyme, lactase. It is not hydrolyzed by maltase, sucrase, or diastase. It differs markedly from the other sugars in the great ease with which it undergoes lactic acid and butyric acid fermentations.

Pictet and Vogel[10, 11] report the synthesis of lactose by heating, for half an hour in a 15 millimeter vacuum at 175°, an equimolecular mixture of β-glucose and β-galactose in the presence of zinc chloride as a catalyst. The residue was freed from chlorides, acetylated, and the acetyl products saponified with sodium methylate, resulting in the isolation of a disaccharide identical with lactose in optical rotation, osazone, acetate, and nitrate.

Kunz and Hudson[12] report the conversion of lactose into a new disaccharide, neolactose, by treating lactose octa-acetate with active aluminum chloride. In this way they obtained a chloracetyl derivative of lactose, and about 30 per cent of the chloracetyl derivative of a new sugar, neolactose. The new derivative was separated by fractional crystallization, and its properties and hydrolytic products determined. The galactose radical was unchanged, pure galactose being recovered upon the hydrolysis of neolactose. The *d*-glucose radical had been changed to *d*-altrose, as shown by the preparation of *d*-altronic acid and *d*-altrose phenylosazone, and by the isolation of *d*-altrose itself. Apparently both the α- and β-carbon atoms had been altered in this transformation.

The fact that lactose occurs to such a large extent in milk whey makes this sugar potentially available in very large quantities.

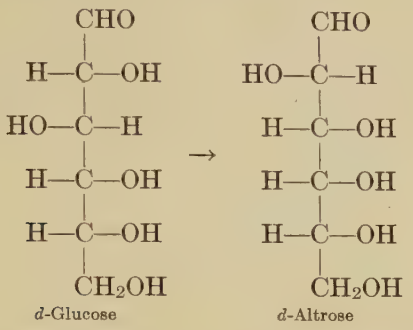
Cellobiose or cellose is a disaccharide obtained by the partial hydrolysis of either cotton or wood cellulose. Its only use at the present time is as a special sugar for bacterial culture media, where it is sometimes

[10] Pictet, A., and Vogel, H., Synthèse du lactose, *Compt. rend.*, 185 : 332–334 (1927).

[11] Pictet, A., and Vogel, H., Synthèse du lactose, *Helv. Chim. Acta*, 11 : 209–215 (1928).

[12] Kunz, A., and Hudson, C. S., Relations between Rotatory Power and Structure in the Sugar Group. XV. Conversion of Lactose to Another Disaccharide, Neolactose. The Chloro-Hepta-Acetate and Two Octa-Acetates of Neolactose, *J. Am. Chem. Soc.*, 48 : 1978–1984 (1926).

used to identify particular types of bacteria. It is utilized by *Aerobacter aerogenes*, but is not attacked by *B. coli*, and is therefore specific in differentiating these two types of organisms.



Peterson and Spencer[13] have recently described a method whereby relatively large yields of cellobiose can be fairly readily obtained.

Melibiose, a glucose-galactoside, is obtained together with fructose from the trisaccharide, raffinose. It apparently differs from lactose in the hydroxyl group of glucose which is united to the galactose radical. Pictet and Vogel[14] report the synthesis of melibiose by converting glucose into a polymer, " diglucosan " by heating glucose in vacuo in the presence of zinc chloride. Galactose was similarly converted into " digalactosan." When a mixture of " diglucosan " and " digalactosan " was heated with zinc chloride, a glass-like solid was obtained, from which by appropriate technic a disaccharide was isolated, which they state to be identical in physical and chemical properties with melibiose.

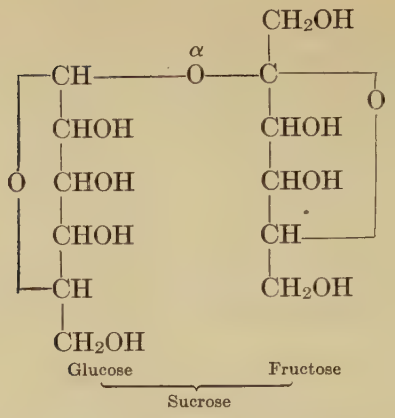
The Non-Reducing Disaccharides.—The principal non-reducing disaccharide is sucrose which is α-glucose-α-(γ)-fructoside. McOwan,[15] and Haworth and Hirst [16] have apparently definitely settled the structure of sucrose, the glucose radical containing the δ-oxide ring, the fructose radical the γ-oxide ring, the union being that of an α-fructoside, as shown in the following formula:

[13] Peterson, F. C., and Spencer, C. C., A Note Concerning a New Method for the Production of Cellobiose from Cellobiose Octa-Acetate, *J. Am. Chem. Soc.*, 49 : 2822–2825 (1927).

[14] Pictet, A., and Vogel, H., Synthèse du melibiose, *Helv. Chim. Acta*, 9 : 806–809 (1926).

[15] McOwan, G., The Constitution of Sucrose, Pt. I. Oxidation of Tetramethyl γ-Fructose. Pt. II. Evidence Contributed by the Oxidation of *d*-Arabinose, *J. Chem. Soc.*, London, July ,1926, 1737–1750.

[16] Haworth, W. N., and Hirst, E. L., The Structure of Fructose, γ-Fructose, and Sucrose, *J. Chem. Soc.*, London, July, 1926, 1858–1868.



The above formula explains (1) why sucrose forms an octa-acetate, *i.e.*, there are eight free —OH groups, (2) why it does not form a phenylhydrazone, inasmuch as there are no " free " or " potential " aldehyde groups, (3) why it does not exhibit mutarotation, since it cannot form an aldehyde without hydrolysis, and (4) why it does not reduce Fehling's solution, inasmuch as it possesses no ketonic or aldehydic properties.

Many organic chemists have attempted the synthesis of sucrose. Pictet and Vogel[17, 18] have only recently announced the achievement of this synthesis. They note that commercial fructose is an equilibrium mixture of the β- and γ-forms, and realized that in order to synthesize sucrose they must isolate a derivative of γ-fructose which would condense with a glucose radical or glucose derivative. Accordingly they acetylated fructose, prepared either from invert sugar or from inulin, and fractionally crystallized the tetra-acetates. After removing all of the normal fructose tetra-acetate, a non-crystallizable fraction still remained. This was dried, yielding an amorphous " glass," a solution of which reduced Fehling's solution at 40° C. and showed other properties indicating that it was in reality the tetra-acetate of γ-fructose. Of this tetra-acetate, 6.5 grams were obtained from 100 grams of ordinary fructose, indicating an equilibrium mixture of 97 parts of the normal fructose to 3 parts of γ-fructose in the original sugar.

An equimolecular mixture of the γ-fructose tetra-acetate and *d*-glucose tetra-acetate was added to dry chloroform in the presence of zinc chloride as a catalyst. After some time, phosphorus pentoxide was added to the mixture and the mixture shaken fifteen hours. The

[17] Pictet, A., and Vogel, H., Synthèse du saccharose, *Compt. rend.*, 186 : 724–727 (1928).

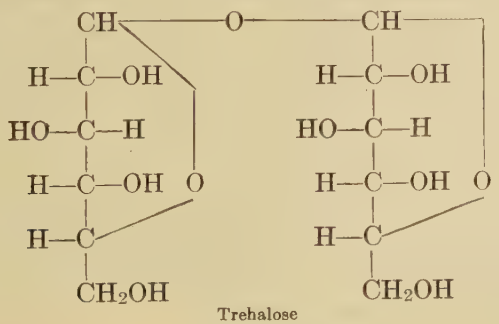
[18] Pictet, A., and Vogel, H., Synthèse du saccharose, *Helv. Chim. Acta*, 11 : 436–442 (1928).

supernatant liquid was removed, evaporated to dryness in vacuo, and the resulting syrup crystallized from hot alcohol. Sucrose octa-acetate separated. The octa-acetate was saponified at low temperature with sodium methylate, yielding crystals of sucrose identical in all of their properties with the natural sucrose.

Sucrose is very generally distributed throughout the plant kingdom, the commercial sugar being derived chiefly from the sugar cane and the sugar beet. The development of the sugar beet industry originated because of the blockade of France during the Napoleonic wars. Napoleon offered a prize for a new source of sugar which would make France independent of the other nations for its sugar supply. At that time, the sugar beet contained only approximately 8 per cent of sucrose. Through the science of plant breeding, aided by chemical analysis, strains of sugar beets have been developed so that at the present time beets which are grown for sugar average from 16 to 18 per cent of sucrose. In some instances, under exceptionally favorable conditions the sucrose content may become as great as 23 per cent. In 1908 the average sugar content of all the sugar beets delivered to the German sugar beet factories was 17.63 per cent. One German factory, during 1908, working with beets averaging 17.10 per cent of sugar actually recovered as crystalline commercial sucrose, 16.64 per cent of the weight of the beet, losing only 0.46 per cent in the extraction and the refining process. This is a striking example of the effect of exact chemical control.

An interesting sucrose preparation is viscogen, prepared by saturating a 50 per cent solution of sucrose with calcium hydroxide and filtering off the excess of calcium hydroxide. This alkaline solution when added in small amounts to cream causes it to " whip " much more readily and allows one to prepare " whipped cream " from ordinary " coffee cream." No adequate explanation has been offered for the physical chemistry of this process.

Trehalose, an α-glucose-α-glucoside, is the only other important non-reducing disaccharide. Its formula is apparently,



Trehalose

It should be noted that the above formula is symmetrical. Trehalose is found widely distributed in the fungi. Inasmuch as they are non-chlorophyll bearing, they form no starch and store trehalose as a reserve food supply. Trehalose does not show mutarotation and forms no osazone.

Sugars of the Formula, $C_{18}H_{32}O_{16}$.—In the trisaccharides, which on hydrolysis yield three monosaccharides, we have again the reducing and non-reducing types.

I. Reducing Trisaccharides

Mannotriose	Glucose-galactose-galactose
Rhaminose	Glucose-rhamnose-rhamnose
Robinose	Rhamnose-rhamnose-galactose

II. Non-reducing Trisaccharides

Raffinose	Galactose-glucose-fructose
Gentianose	Glucose-glucose-fructose
Mellezitose	Glucose-glucose-fructose

Robinose has only recently been found by Charaux[19] in the glucoside, robinine, obtained from the fresh flowers of *Robinia pseudo-acacia* D.C. Charaux reports that the sugar reduces Fehling's solution in the cold, which would be an indication that at least one of the sugar radicals was present as the γ-sugar.

Raffinose is the best known of the trisaccharides. It occurs in appreciable amounts in the sugar beet, and special corrections have to be made to the readings of a polarimeter in order to determine accurately sucrose in the presence of raffinose. Insofar as its chemical reactions are concerned, it is quite similar to sucrose. Its structure is that of two disaccharides combined in one molecule with the glucose radical in common (cf. Haworth, Hirst, and Ruell[20]).

Fructose-O-glucose-O-galactose
(1) (2) (3)

Sucrose Melibiose
(4) (5)

[19] Charaux, C., Sur le dédoublement biochimique du Robinoside (Robinine).—, Robinose, nouveau triose, provenant de ce dédoublement, *Bull. soc. chim. biol.* 8 : 915–923 (1926).

[20] Haworth, W. N., Hirst, E. L., and Ruell, D. A., The Constitution of Raffinose, *J. Chem. Soc.*, London, 123 : 3125–3131 (1923).

Here again we have a striking example of the specificity of enzyme action.

1. The strong mineral acids hydrolyze raffinose to its constituent monosaccharides, fructose, glucose, and galactose.

2. Weak acids (or a low hydrogen ion concentration) hydrolyze only the sucrose part of a molecule, yielding fructose (1) and melibiose (5), as the hydrolytic products. Here we have the potential sucrose residue, showing the same ease of hydrolysis that characterizes sucrose.

3. Sucrase yields as hydrolytic products, fructose (1) and melibiose (5), *i.e.*, sucrase hydrolyzes only the sucrose residue.

4. Emulsin, in contrast to sucrase, hydrolyzes the melibiose portion of the molecule, yielding galactose (3) and sucrose (4).

5. Bottom yeasts contain both melibiase and sucrase, so that they hydrolyze raffinose completely to fructose, glucose, and galactose.

In addition to being found in the sugar beet, raffinose is frequently found in the higher plants and in fungi. It has been reported to be present in certain invertebrates. Cottonseed meal contains about 8 per cent of raffinose and is the usual material from which this sugar is prepared.

Raffinose is valueless as a food until after hydrolysis, and in man such hydrolysis as does take place is apparently due to the bacterial action in the large intestine and not to any enzymes which we normally possess (cf. Kuriyama and Mendel[21]).

Haworth and Wylam[22] have carried out a similar study to determine the structure of gentianose. This sugar is apparently,

$$\underbrace{\underset{(1)}{\text{Glucose}} + \underset{(2)}{\text{glucose}} + \underset{(3)}{\text{fructose}}}_{\substack{\text{Gentiobiose} \\ (4)}} \quad \underbrace{\qquad\qquad}_{\substack{\text{Sucrose} \\ (5)}}$$

It is accordingly composed of two disaccharides, sucrose and gentiobiose, holding one glucose radical in common.

Melezitose is an exceedingly rare sugar. It is composed of three molecules of glucose and can be hydrolyzed to yield glucose and a disaccharide, turanose. Melezitose occurs in the sap of the larch, Douglas fir, scrub pine, etc., and under certain conditions, especially following

[21] Kuriyama, S., and Mendel, L. B., The Physiological Behavior of Raffinose, *J. Biol. Chem.*, 31 : 125–147 (1917).

[22] Haworth, W. N., and Wylam, B., The Constitution of the Disaccharides. Pt. IX. Gentiobiose: Its Identity with Amygdalin Biose, *J. Chem. Soc.*, London, 123 : 3120–3125 (1923).

attacks by sucking insects, these trees exude drops of sweet sap which harden into a more or less true " manna." In periods when nectar is scarce, bees may store this " honey dew " as a substitute for honey. This was the case in the dry summer of 1917 in certain portions of the eastern United States when the clover blossoms quickly dried because of drought. The following winter, many bee colonies throughout this area died. The " honey " in these hives had crystallized. The Bureau of Chemistry of the United States Department of Agriculture undertook an investigation as to the cause of the death of the bees and found the " honey " to contain very large quantities of melezitose. From this source they prepared several kilos of this rare sugar (cf. Hudson and Sherwood,[23] and Wherry[24]).

Even if the " honey " had not crystallized, the bees would nevertheless have died, for they possessed no enzymes to hydrolyze the melezitose and therefore could not use as food the " honey " which they had stored.

Sugars of the Formula, $C_{24}H_{42}O_{21}$.—The only tetrasaccharide known is stachyose, isolated by Planta and Schulze[25] from the tubers of *Stachys tubifera*. On hydrolysis it yields one molecule of glucose, one molecule of fructose, and two molecules of galactose. On mild hydrolysis the molecule of glucose is split off, yielding a trisaccharide, mannotriose, containing one molecule of fructose and two molecules of galactose.

[23] Hudson, C. S., and Sherwood, S. F., The Occurrence of Melezitose in Honey, *J. Am. Chem. Soc.*, 42 : 116–125 (1920).

[24] Wherry, E. T., The Crystallography of Melezitose, *J. Am. Chem. Soc.*, 42 : 125–128 (1920).

[25] Planta, A. von, and Schulze, E., Ueber ein neues krystallisirbares Kohlenhydrat, *Ber.* 23 : 1692–1699 (1890).

CHAPTER XXV

THE POLYSACCHARIDES

The polysaccharides are, in general, characterized by forming colloidal sols rather than true solutions. The molecular weight is invariably large, corresponding in some instances at least to the molecular weights of the proteins. Many of the polysaccharides are strongly hydrophilic. They may be classified as has already been indicated in Chapter XXI, or the following simple classification may be used.

I. Starches or hexosans
   - Glucosans
   - Fructosans
   - Mannosans
   - Galactosans
   - Amyloids, etc.

II. Gums or mucilages
   - Pentosans
   - Natural gums
   - Pectins
   - Plant mucilages

III. Celluloses
   - Hemi-celluloses
   - True celluloses
   - Compound celluloses

It is highly probable that very few of the groups mentioned above accurately express the nature of the material, for many of the polysaccharides contain non-carbohydrate radicals.

The Starches or Hexosans, $(C_6H_{10}O_5)_x$.—These polysaccharides on hydrolysis yield hexoses. The chief hexosan is starch or amylum. It forms the reserve food material of most plants and is the source of energy for most plant embryos. Starch is to plants what the fats are to animals. Starch occurs in the cells in granules having characteristic striations. These striations and the size and shape of the granules are

more or less characteristic of many plant forms and may be used as a microscopical means of identifying the biological origin of the starch, (cf. Reichert[1]).

Amylases or diastase hydrolyze the starch to dextrins, and these in turn to maltose which is the end product of diastatic action. Acids or maltase continue the hydrolysis to glucose. Blake,[2] and Davis,[3] and Daish[4] have presented excellent discussions of enzymes in relation to starch and sugar.

The structure of starch is unknown. Various degradation products have been given names, and it has been suggested that the following scheme represents the course of hydrolysis:

$$\text{Starch} \rightarrow \text{amylodextrin} \rightarrow \begin{matrix} \text{maltose} \\ + \\ \text{erythrodextrin} \end{matrix} \rightarrow \begin{matrix} \text{maltose} \\ + \\ \text{achroodextrin} \end{matrix} \rightarrow \text{maltose} \rightarrow \text{glucose.}$$

The amylodextrins, erythrodextrins, and achroodextrins are differentiated by the color which they give with iodine, *i.e.*, blue → purple → red-brown. It has been suggested that these color changes do not necessarily mean anything more than a change in size of the starch particle and that in reality instead of forming distinct compounds, the diastase simply peptizes the starch granules, the whole series of color reactions with iodine being colloidal phenomena depending on the degree of dispersion, in the same way that the color of colloidal gold sols changes with size of particle, being red at the smaller sizes and blue to black at the larger sizes (cf. Herzfeld and Klinger,[5, 6] and Stocks[7]).

[1] Reichert, E. T., The Differentiation and Specificity of Starches in Relation to Genera, Species, etc., Stereochemistry Applied to Protoplasmic Processes and Products, and as a Strictly Scientific Basis for the Classification of Plants and Animals, Pts. I and II, Carnegie Institution of Washington, Pub. 173, Washington, D. C. (1913).

[2] Blake, J. C., On the Digestibility of Bread, I. Salivary Digestion *in Vitro*, *J. Am. Chem. Soc.*, 38 : 1245–1260 (1916).

[3] Davis, W. A., The Distribution of Maltase in Plants. I. The Function of Maltase in Starch Degradation and Its Influence on the Amyloclastic Activity of Plant Materials, *Biochem. J.*, 10 : 31–48 (1916).

[4] Daish, A. J., The Distribution of Maltase in Plants. II. The Presence of Maltase in Foliage Leaves. III. The Presence of Maltase in Germinated Barley, *Biochem. J.*, 10 : 49–55, 56–76 (1916).

[5] Herzfeld, E., and Klinger, R., Zur Chemie der Polysaccharide, *Biochem. Z.*, 107 : 268–294 (1920).

[6] Herzfeld, E., and Klinger, R., Berichtigung und Ergänzung zu unserer Arbeit; "Zur Chemie der Polysaccharide," *Biochem. Z.*, 112 : 55–60 (1920).

Some authors claim that when starch is gelatinized by heat in the presence of water, two products are formed, (1) starch cellulose (α-amylose), insoluble in water, and (2) granulose (β-amylose) which is water-soluble. Others call the outer layer " amylopectin." Possibly there is a chemical difference in the outer layer and the inner material of the starch grains. Taylor and Iddles[8] report a method for the separation of α- and β-amyloses. They note that on electrolysis of a sol containing α- and β-amyloses, the α-amylose migrates to the positive electrode, whereas the β-amylose remains dispersed in the solution. They similarly found that β-amylose could be ultrafiltered from the α-amylose micelles, yielding clear sols of β-amylose. According to their findings, corn starch contains from 84.4 to 88.5 per cent of β-amylose and from 15.6 to 11.5 per cent of α-amylose. Rice starch, according to Taylor and Iddles, contains 83.0 to 84.1 per cent of β-amylose, and potato starch 98.1 to 98.3 per cent of β-amylose.

Other workers have expressed the belief that the starch granules are more or less uniform throughout and that the distinction which has been made between α- and β-amyloses is due to the fact that the outer layers of the granule are more or less resistant to chemical action, on the hypothesis that the outer layers are somewhat more dehydrated.

The gelatinization of starch by heating with water has a very marked temperature coefficient. In general, no reaction will be observed until about 55° C. When starch is viewed under the microscope and this temperature has been reached, it will be noted that a few granules swell to a relatively enormous volume. As the temperature slightly increases, the number of swollen granules rapidly increases until all have swollen and a considerable number have burst. The point at which this transformation takes place is known as the gelation temperature or " thickening point " and has been reported as being more or less characteristic of the plant source from which the starch is derived.

Dox and Roark,[9] however, in a study of 13 different varieties of maize, report a range in the gelation temperature from 64° to 71° C. Potato starch, as a rule, gelatinizes at a lower temperature than most other starch. Sago starch and rice starch, as a rule, require a higher temperature.

[7] Stocks, H. B., Colloid Chemistry of Starch, Gums, Hemicelluloses, Albumin, Casein, Gluten, and Gelatine, First Report on Colloid Chemistry, *Brit. Assoc. Adv. Sci.*, pp. 46–78 (1917).

[8] Taylor, T. C., and Iddles, H. A., Separation of the Amyloses in Some Common Starches, *Ind. Eng. Chem.*, 18 : 713–717 (1926).

[9] Dox, A. W., and Roark, G. W., Jr., The Determination of Gelatinization Temperatures of Starches by Means of an Electrically Heated Chamber on the Microscope Stage, *J. Am. Chem. Soc.*, 39 : 742–745 (1917).

Alsberg and Griffing[10] report that grinding starch in a ball mill causes it to disperse in water in a manner similar to " soluble starch." In a study of the gelatinization of starch, Alsberg[11] finds that, contrary to most of the statements in the literature, starch granules when heated swell but *do not burst*, although the potato starch granules do disintegrate to some extent. He believes that it is because of this disintegration that gelatinized potato starch does not form as viscous a suspension as do the other starches. In investigating the swollen granules under the microscope, Alsberg noted that the granules appeared to be composed of a sac-like structure filled with a fluid. When tannin was allowed to diffuse into such swollen granules, it formed particles which exhibited strong Brownian movement, indicating that the interior of the granules is either a sol or a solution, not a gel. When such starch pastes were ground, there was a pronounced decrease in viscosity, which Alsberg explains as being due to a rupture of the envelope of the starch granule, allowing the fluid interior to escape. When dry starch was ground in a ball mill, a similar disintegration took place, so that when the ground starch was later heated with water, a thick syrup was formed instead of a gel.

Alsberg notes three factors as being responsible for the gelatinization of starch by heat.

1. The ease with which the anatomical structure is softened by moist heat.
2. The inherent swelling power of the granule substance.
3. The relation of the mass of the swelling substance to the surface area of the granule.

The larger the granule, the greater is the mass of the swelling substance which exerts pressure upon a unit area of the granule surface. Therefore, a large granule will swell more rapidly than a small one, and it should swell sooner if the temperature is raised. Alsberg believes that there is no such physical constant as a " gelatinization temperature " but that instead, there is only a " gelatinization range."

In the paper referred to, Alsberg suggests that gelatinized starch pastes are suspensions and not true colloidal sols, so that the setting of the gelatinized starch to a more or less rigid solid is not analogous to the gelation of a substance, such as gelatin. He states, " Boiled starch forms paste only when the ratio of starch to water is so chosen that the swollen granules occupy most of the volume of the system and therefore touch or jostle one another. The viscosity of a still warm, swollen

[10] Alsberg, C. L., and Griffing, E. P., Effect of Fine Grinding upon Flour, *Cereal Chem.*, 2 : 325–344 (1925).

[11] Alsberg, C. L., Studies upon Starch, *Ind. Eng. Chem.*, 18 : 190–193 (1926).

starch suspension is for the greatest part the resultant of this jostling. . . . . The microscope shows that warm boiled starch granules are easily deformable and elastic. They can easily be made to move past one another, to flow. In paste they are much less easily deformable, much less elastic. At lower temperatures apparently a portion of the rigidity of the original starch is recovered. Possibly, too, the granule surfaces are more sticky, favoring their agglutination, one with the other."

Alsberg and Griffing[12] report the crystallization of starch by autoclaving at 150° to 160° C. the clear solution prepared from starch which had been ground in a ball mill. They report the starch crystals as separating in needles which give typical iodine reactions for starch, the needles being only slightly soluble in cold water but much more readily soluble in hot water.

The quality of starch, its sizing properties, and its viscosity are dependent upon its degree of colloidal dispersion. There is an optimum for all of these properties in most commercial work. The sago, tapioca, and cassava starches usually yield more gelatinous sols than do the ordinary grain starches. One of the best recent papers on the colloidal properties of starches is that of Farrow and Lowe[13] (cf. also Rask and Alsberg[14]). Samec also has published an excellent series of papers under the general title, " Studien ueber Pflanzenkolloide," most of which have appeared in the *Kolloide Beihefte* (cf. also " Kolloidchemie der Stärke" [15]).

Raw starch is appreciably hydrophilic, so that at the usual atmospheric humidity it contains 13–15 per cent of moisture (cf. Nelson and Hulett[16]).

It is generally recognized that when starch is hydrolyzed, it is not quantitatively converted into glucose. An unfermentable residue always remains. We have already noted the presence of gentiobiose (isomaltose) in the products of the acid hydrolysis of starch. Whether or not other groupings occur in appreciable amounts is still an open question. Spoehr believes that starch always contains more or less pentose material. There is a small amount of mineral matter (ash, about 0.22 per cent in potato starch, 0.40 per cent in sago starch, 0.23

[12] Alsberg, C. L., and Griffing, E. P., The Crystallization of Starch, *Proc., Soc. Exp. Biol. Med.*, 23 : 728–730 (1926).

[13] Farrow, F. D., and Lowe, G. M., Flow of Starch Paste through Capillary Tubes, *J. Text. Inst.*, 14 : 414–440 (1923).

[14] Rask, O. S., and Alsberg, C. L., A Viscosimetric Study of Wheat Starches, *Cereal Chem.*, 1 : 7–26 (1924).

[15] Samec, M., Kolloidchemie der Stärke, Theodor Steinkopff, Dresden and Leipzig (1927).

[16] Nelson, O. A., and Hulett, G. A., The Moisture Content of Cereals, *J. Ind. Eng. Chem.*, 12 : 40–45 (1920).

per cent in wheat starch), and phosphoric acid is always a constituent of the ash. It is probably present in organic combination in the starch, although it may be that it is simply adsorbed on the surfaces. Taylor and Nelson[17] have made a careful study of starch and find that a fat is always associated with the starch in the granule.

Taylor and Lehrman,[18] and Taylor and Werntz[19] report oleic, linolic, and palmitic acids as constituents of corn starch. The yield of fatty acids amounted to 0.5 to 0.6 per cent of the weight of the starch. The three acids were present in approximately the following amounts, palmitic acid 24 per cent, oleic acid 40 per cent, and linolic acid 36 per cent, of the total. These studies have opened up a new field for research in the polysaccharides.

It is impracticable to even casually discuss the field of starch chemistry. An excellent survey of this field has recently been presented under the editorship of Walton.[20]

The Mechanism of the Transformation of Glucose to Starch in the Plant.—The value of potassium as a fertilizer is at least in part due to its functioning importantly in starch formation. Potassium is apparently necessary in the reaction whereby glucose, the product of photosynthesis, is transformed into starch, the reserve food material. The nature of this reaction, however, is almost wholly unknown. From the standpoint of physical chemistry, it is essential that the plant transform the glucose into a higher polymer, such as starch, as a reserve food supply. In so long as glucose is present in solution, the osmotic pressure within the cells must be at least proportional to the glucose concentration. When, however, the glucose is converted into the relatively insoluble and osmotically-inert starch, a large reserve supply of food materials may be stored without unduly affecting the osmotic relations of the tissues.

Chapman[21] has studied the enzymatic relationships in certain plants and suggests a mechanism for the transformation of glucose to starch within the cells. He notes, " The guard-cell starch of those plants which do not contain any other starch may be regarded as formed by a

[17] Taylor, T. C., and Nelson, J. M., Fat Associated with Starch, *J. Am. Chem. Soc.*, 42 : 1726–1738 (1920).

[18] Taylor, T. C., and Lehrman, L., The Unsaturated Fatty Acids Associated with Corn Starch, *J. Am. Chem. Soc.*, 48 : 1739–1743 (1926).

[19] Taylor, T. C., and Werntz, J. H., Properties of Corn Starch. Removal of Combined Fatty Acids, *J. Am. Chem. Soc.*, 49 : 1584–1588 (1927).

[20] Walton, R. P., A Comprehensive Survey of Starch Chemistry, Vol. I, The Chemical Catalog Company, Inc., New York (1928).

[21] Chapman, R. E., The Carbohydrate Enzymes of Some Starch-free Monocotyledons, *Biochem. J.*, 18 : 1388–1400 (1924).

mechanism which later disappears. The snowdrop has no maltase. But starch cannot be formed from glucose without maltase (if enzymes are true catalysts), so that presumably in the young leaf, the whole apparatus—maltase, dextrinase and amylase—required to convert glucose into starch is present in the guard-cells. Starch is formed, and afterwards the maltase disappears. Thus no more starch can be formed, but equally the starch already formed cannot be hydrolyzed further than maltose. The series of changes

$$\text{Starch} \rightleftarrows \text{Maltose}$$

under the influence of amylase and dextrinase would form an ideal mechanism for controlling the opening of the stomata if the reaction is

$$\text{Starch} \rightarrow \text{Maltose}$$

when the plant has plenty of water and transpiration is not too intense, and

$$\text{Starch} \leftarrow \text{Maltose}$$

when transpiration is high and water supplies are failing. But these are exactly the circumstances required, because the full equation is

$$\text{Starch} + \text{Water} \rightleftarrows \text{Maltose},$$

and a high water content in the leaf would cause the reaction to go to the right. The maltose thus formed would increase the soluble contents of the guard-cells, water would be taken in by osmosis until turgidity was reached, and the stomata would open. If water were lacking, the reaction would go to the left, maltose would be replaced by insoluble starch, the soluble contents of the guard-cells would be lessened, water would be lost by osmosis, the guard-cells would no longer be turgid and the stomata would close. This system of amylase and dextrinase with no maltase might be described as a " chemical appendix," a relic of an earlier state, but in this case the appendix is still functional and useful. The data seem to indicate that there is only a small quantity of diastase in the snowdrop, which would be quite compatible with this theory.

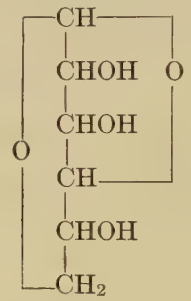
" Similarly, the onion and the leek cannot form starch in the green leaf, because the complete starch-forming machinery is again lacking; the onion lacks dextrinase and the leek lacks amylase.

" The dock can form starch because it contains the full equipment of maltase, dextrinase and amylase."

Dextrins (Sometimes called British Gum).—This is a collective name for a group of intermediate products formed by the partial hydrolysis of starch. They are distinguished from starch by being soluble in cold water and from sugars by being precipitated by alcohol. They have a

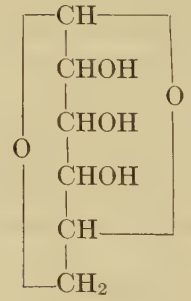
high dextro-rotation. Some idea as to the degree of hydrolysis which the starch has undergone can be secured from a determination of the gold number of the resulting dextrin. The manufacture of dextrin is usually carried out by autoclaving starch in the presence of a weak acid, *e.g.*, 5 to 15 pounds pressure with one per cent citric acid. Dextrins are largely used as adhesives. The " glue " of the postage stamps, envelopes, etc., is dextrin.

Irvine and Oldham,[22] as well as Pictet,[23, 24] have prepared various polymers of glucose by treating starch with a catalyst, or by fusing it *in vacuo*, or by dry distillation. Irvine and Oldham note that by dry distillation starch yields β-glucosan or 1–6-anhydroglucose. They give the formula of this compound as shown in (*A*),



(*A*)

However, since the publication of this paper, it has been shown that the structure of glucose is that of a δ-oxide rather than a γ-oxide, as shown in (*A*). The formula, accordingly, is more probably that shown in (*B*).



(*B*)

[22] Irvine, J. C., and Oldham, J. W. H., Polymerisation of β-Glucosan. The Constitution of Synthetic Dextrins, *J. Chem. Soc.*, London, 127 : 2903–2922 (1925).

[23] Pictet, A., Sur la transformation de la lévoglucosane en dextrine, *Helv. Chim. Acta*, 1 : 226–230 (1918).

[24] Pictet, A., Sur la polymérisation de la glucosane, *Helv. Chim. Acta*, 4 : 788–795 (1921).

It is possible by the methods outlined by Pictet or by Irvine and Oldham to prepare definite anhydro-products corresponding to 2, 3, 4, 5, or more glucose molecules, which have been designated as diglucosan, triglucosan, tetraglucosan, heptaglucosan, and polyglucosan. Perhaps when these are sufficiently studied, we may be able to prove the exact structure of certain naturally-occurring polysaccharides.

Glycogen or "Animal Starch."—Glycogen is the reserve carbohydrate of the muscles and especially of the liver. It is not found in green plants but is present in certain fungi, especially in yeast, as a reserve foodstuff. It is a white, amorphous powder, soluble in cold water to an opalescent, colloidal solution which gives a red-brown color with iodine, is precipitated by alcohol, does not reduce Fehling's solution, and is hydrolyzed to glucose.

Levulosans, Including Inulin and Inulides.—These are hydrolyzable to fructose. Inulin is a white, more or less crystalline powder, readily soluble (colloidal) in hot water, but slightly soluble in cold water (it can be recrystallized by this process), does not gelatinize on heating, does not turn blue with iodine, is not hydrolyzed by amylase or ptyalin, but is hydrolyzed by inulase. The dahlia tubers contain 10 to 12 per cent of inulin (cf. Willaman,[25] Irvine and Steele,[26] Irvine, Steele, and Shannon,[27] and Colin and Cugnac[28]).

Mannosans.—The straw of cereals, the leaves and, to some extent, the wood of trees, and a part of the roots of certain plants are the usual sources of mannosans. Mannosans are apparently a structural component of plants. The mannosans closely resemble cellulose in many of their properties, except that upon acid hydrolysis they yield mannose rather than glucose.

Galactosans.—These resemble mannosans, except that upon hydrolysis they yield galactose. They are widely distributed in nature as a structural element of plants (cell walls, etc.). Insofar as is known, there is no specific enzyme by which their hydrolysis can be accomplished.

Mucilages.—Some of the better known mucilages are carrageen or "Irish moss," and agar-agar. They are the structural components of the cells of algae, and are prepared from "sea weeds." They are odorless, tasteless, and swell strongly in water but do not dissolve. When

[25] Willaman, J. J., The Preparation of Inulin, with Special Reference to Artichoke Tubers as a Source, *J. Biol. Chem.*, 51 : 275–283 (1922).

[26] Irvine, J. C., and Steele, E. S., The Constitution of Polysaccharides. Pt. I. The Relationship of Inulin to Fructose, *J. Chem. Soc.*, London, 117 : 1474–1489 (1920).

[27] Irvine, J. C., Steele, E. S., and Shannon, M. I., The Constitution of Polysaccharides. Pt. IV. Inulin, *J. Chem. Soc.*, London, 121 : 1060–1078 (1922).

[28] Colin, H., and Cugnac, A. de, Les levulosanes des Graminées : graminine et triticine, *Bull. soc. chim. biol.*, 8 : 621–630 (1926).

heated with water, they form sols which upon cooling set to a rigid gel. Such gels do not "melt" as readily as do gelatin gels. A one per cent agar gel is a fairly rigid gel. These gels are not liquefied by organisms which digest gelatin; consequently they are very useful in bacteriological laboratories. The animal body does not possess enzymes capable of digesting agar; consequently it cannot be utilized as a food and is sometimes prescribed to furnish bulk in cases of constipation. The presence of agar in the food can be readily determined by finding diatom shells in the ash, for the agar always contains diatom skeletons.

Agar and carrageen are both sulfuric acid esters, where the ester group is a complex polysaccharide. The gelatinization of agar is the gelatinization of a salt of an agar sulfuric acid (agar—O—SO_2—OH). This salt may be metallic, as sodium, potassium, magnesium, calcium, etc., or it may be an organic base, such as aniline or an alkaloid, and satisfactory gels will be formed. The " agar acid " itself will not gelate, and is so strongly acid that it undergoes auto-hydrolysis upon heating. A one per cent solution of the free agar acid has a *p*H of approximately 2.0 (cf. Hoffman and Gortner,[29] Haas and Hill,[30] and Haas[31]).

On acid hydrolysis agar-agar yields galactose, or a carbohydrate isomeric with galactose. Besides 20 to 28 per cent of galactan, there is evidence for the presence of mannans and fucosans. The question as to the exact composition of the carbohydrate portion of agar or carrageen is still more or less uncertain, although these two mucilages have been worked with more than any others.

Gums.—Most of the gums are not represented by the formula, $(C_6H_{10}O_5)_x$, but are in reality more or less glucoside-like compounds consisting of hexoses or pentoses (or both), combined with other substances, generally complex acids. On hydrolysis they usually yield galactose, arabinose, or xylose, either alone or in mixtures. Certain gums have yielded an acid which apparently has the formula $C_{23}H_{38}O_{22}$. Gum acacia or gum arabic is probably the best known example of this group of compounds.

Thomas and Murray[32] have prepared the free acid of gum arabic, using the technic that was used by Hoffman and Gortner to prepare the

[29] Hoffman, W. F., and Gortner, R. A., The Electrodialysis of Agar. A Method for the Preparation of the Free Agar-Acid, *J. Biol. Chem.*, 55 : 371–379 (1925).

[30] Haas, P., and Hill, T. G., On Carrageen. *Chondrus Crispus*, *Ann. Applied Biol.*, 7 : 352–362 (1921).

[31] Haas, P., On Carrageen (*Chondrus crispus*). II. On the Occurrence of Ethereal Sulphates in the Plant, *Biochem. J.*, 15 : 469–476 (1921).

[32] Thomas, A. W., and Murray, H. A., Jr., A Physico-Chemical Study of Gum Arabic, *J. Phys. Chem.*, 32 : 676–697 (1928).

free agar-acid. They find arabic acid to be a relatively strong acid, a one per cent solution showing a *p*H value of 2.70, which is equivalent to a 0.02 N solution of hydrochloric acid. It is, however, not an ester of either sulfuric acid or of phosphoric acid, since it contains no sulfur, phosphorus, or chlorine. From its titration values with sodium and barium hydroxides, the equivalent weight of the acid appears to be approximately 1200. Gum arabic, therefore, can be looked upon as the calcium or calcium-magnesium salt of arabic acid. Relatively little is known in regard to the exact nature of the carbohydrate groups in such compounds.

Immunologically Specific Polysaccharides.—Various workers [33] have noted that certain bacterial cultures contain a "soluble specific substance" which reacts with antisera to form precipitates. Thus, the fluid cultures of pneumococci contain a substance which is a specific precipitant for anti-pneumococcus serum even when a dilution of the soluble specific substance is as high as 1 : 6,000,000.

The nature of this soluble specific substance has been studied by Heidelberger and Goebel.[34-36] As a result of this work, it appears that the specific material is a polysaccharide containing groupings of an entirely different character from polysaccharides which have been investigated previously. Heidelberger and Goebel, as a result of their study of the polysaccharide isolated from Type III pneumococcus, obtained an acid having the formula, $C_{11}H_{19}O_{10}\cdot COOH$, which upon further hydrolysis yielded glucose and a hexoseuronic acid. They later showed the uronic acid to be glucuronic acid and that the reducing part of the molecule of the aldobionic acid belonged to glucose, the union between the glucose and the glucuronic acid radical being through one of the hydroxyl groups of glucose with the potential aldehyde group of the glucuronic acid, as shown in the formula on the following page.

In this formula, the glucosidal linkage is through the ϵ-carbon atom of glucose. That the sixth carbon atom is involved is not proven. It may well be that this linkage is not ϵ but either α, β, or γ. It is cer-

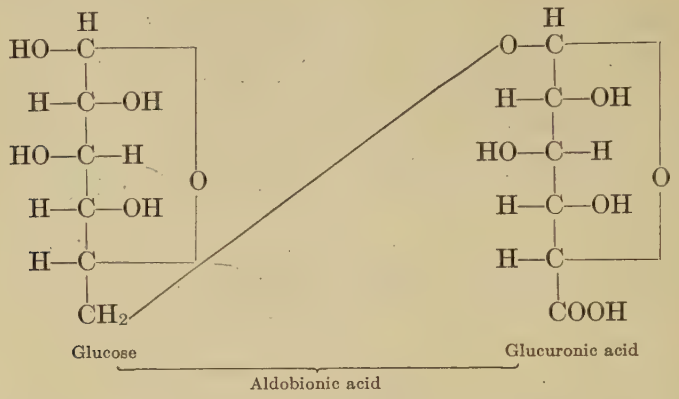
[33] For literature see papers by Heidelberger and Goebel shortly to be cited.

[34] Heidelberger, M., and Goebel, W. F., The Soluble Specific Substance of Pneumococcus. IV. On the Nature of the Specific Polysaccharide of Type III Pneumococcus, *J. Biol. Chem.*, 70 : 613–624 (1926). V. On the Chemical Nature of the Aldobionic Acid from the Specific Polysaccharide of Type III Pneumococcus, *J. Biol. Chem.*, 74 : 613–618 (1927).

[35] Goebel, W. F., The Soluble Specific Substance of Friedländer's Bacillus. IV. On the Nature of the Hydrolytic Products of the Specific Carbohydrate from Type A Friedländer Bacillus, *J. Biol. Chem.*, 74 : 619–629 (1927).

[36] Heidelberger, M., Immunologically Specific Polysaccharides, *Chem. Rev.*, 3 : 403–423 (1927).

tain, however, that it does not involve the terminal, potentially aldehydic, carbon atom.



Goebel gives the formula of the glucuronic acid portion of the molecule as possessing a γ-oxide ring and being therefore a derivative of γ-glucose, rather than possessing the normal δ-oxide ring. Since, however, glucuronic acid on the loss of carbon dioxide yields a pentose of normal structure, it seem probable that the δ-oxide ring is present, as figured.

In a study of the Type A Friedländer bacillus, Goebel finds an aldobionic acid, isomeric with the similar acid obtained from the pneumococcus Type III carbohydrate, composed of glucose and glucuronic acid linked in a manner similar to the formula already given but possessing somewhat different physical properties. He concludes, therefore, that these two sugar acids, one isolated from the pneumococcus, the other from the Friedländer bacillus, differ only in their spatial configurations. In the case of the Friedländer bacillus, a second disaccharide acid of undetermined nature is likewise present, and Goebel expressed the belief that the polysaccharide is built up of three radicals, one of which is the glucose-glucuronic acid radical, another a new disaccharide acid, and the third glucose itself. On this basis the empirical formula of the polysaccharide would be $(C_{30}H_{44}O_{26})_x$.

These studies are particularly important, inasmuch as they appear to have demonstrated that polysaccharides, as well as proteins, are involved in the problem of the chemistry of immunity.

Cellulose.—The term, cellulose, covers a class of compounds found in the woody portion of plants. These compounds are characterized by possessing the formula $(C_6H_{10}O_5)_x$, by being insoluble in water and in all organic solvents, soluble in " Schweitzer's reagent " (ammoniacal

copper hydroxide, cf. Neale[37]), soluble in zinc chloride dissolved in twice its weight of hydrochloric acid, soluble in molten $FeCl_3 \cdot 6H_2O$, and more or less completely hydrolyzed in such solutions to glucose.

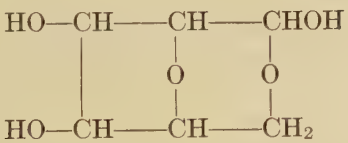
The literature dealing with cellulose is very voluminous. Montonna[38] has prepared a bibliography covering the more important papers dealing with cellulose and cellulose derivatives. Similarly, Hibbert[39] has reviewed the various papers dealing with the structure of the cellulose molecule and has presented a rather extensive bibliography.

Cellulose is relatively inert toward mild chemical reagents. Treated with dilute alkalies there is little immediate reaction. Stronger alkalies, such as 10 per cent sodium hydroxide, form " hydrocellulose " and " mercerize " the fiber. Dilute sulfuric acid forms a " hydrocellulose," while stronger sulfuric acid converts the cellulose fibers into " vegetable parchment." Dilute nitric acid forms " oxycellulose," and concentrated nitric acid forms nitrocellulose.

Irvine and Soutar[40] hydrolyzed cellulose with acid, and on the theory that cellulose is composed wholly of glucose, obtained 85 per cent of the theoretical yield of glucose as the crystalline sugar. They believe that glucose is the only product formed by the hydrolysis of cellulose.

Upon destructive distillation, cellulose yields acetone, acetic acid, and methyl alcohol, indicating the presence of a $-CH_2C{=}O$ grouping in the molecule. The highest nitrate is three NO_2 groups to each six carbon atoms; the highest acetate is three acetyl groups to each six carbon atoms, indicating that only three hydroxyl groups in each molecule of glucose remain uncombined.

Various structural formulae have been suggested to explain the reactions of cellulose. The older formula is that of Green, which is simply a dehydrated glucose molecule.



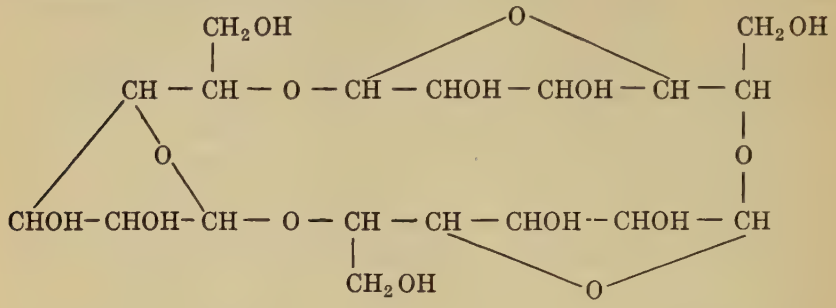
[37] Neale, S. M., The Nature of Solutions of Cellulose in Cuprammonium Hydroxide, *J. Text. Inst.*, 16 : 363–369 (1925).

[38] Montonna, R. E., The Chemistry and Technology of Cellulose. A Bibliographic Review, *Paper Trade J.*, 86 : 61–67 (1928).

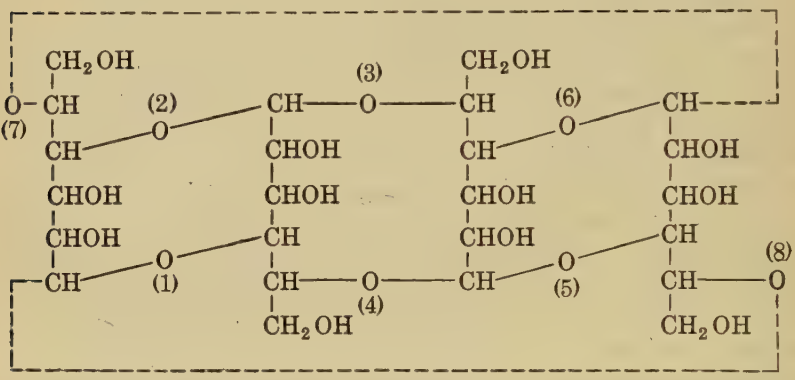
[39] Hibbert, H., Studies on the Chemistry of Cellulose. I. The Constitution of Cellulose, *J. Ind. Eng. Chem.*, 13 : 256–260, 334–342 (1921).

[40] Irvine, J. C., and Soutar, C. W., The Constitution of Polysaccharides. Pt. II. The Conversion of Cellulose into Glucose, *J. Chem. Soc.*, 117 : 1489–1500 (1920).

This formula has been modified by Hibbert and Timm[41] who suggest a formula in which three molecules of glucose are condensed.



Schorger[42] proposes a formula involving four molecules of glucose.



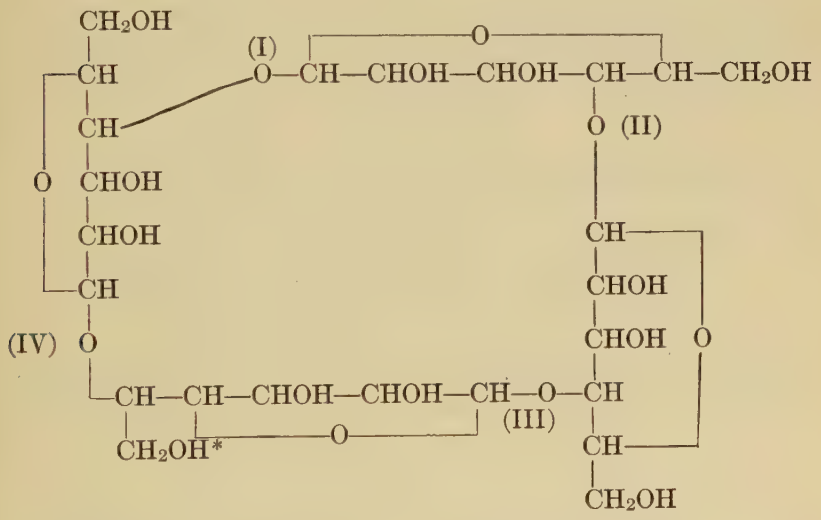
In support of this formula he suggests, " 1. It is capable of yielding only 2, 3, 6-trimethylglucose. 2. It is possible to obtain a theoretical yield of cellobiose octaacetate on acetolysis, but this theoretical yield is highly improbable, since: 3. It is also capable of yielding an isomer of cellobiose, although this isomer would not be maltose. Fission at (1), (2), (4), (5), and (6) would give cellobiose, and at (7), (8), (1), (3), and (4), the isomeride, isocellobiose. There are equal chances for the formation of cellobiose and isocellobiose. Failure to isolate the latter may be due to the greater ease of hydrolysis or acetolysis at (1), (2), (6), and (5) due to the valency angles. 4. Hydrolysis at (2) or (3) would result in a

[41] Hibbert, H., and Timm, J. A., Studies on Cellulose Chemistry. IV. Properties of Gamma-Delta-Dihydroxy-Carbonyl Derivatives and Their Bearing on the Polymerization of Polysaccharides, *J. Am. Chem. Soc.*, 45 : 2433–2439 (1923).

[42] Schorger, A. W., The Constitution of Cellulose, *Ind. Eng. Chem.*, 16 : 1274–1275 (1924).

slight degradation of the molecule but not in the formation of an active carbonyl group; at both (2) and (3) an aldehyde group would be formed, giving a hydrocellulose. The molecule would still be largely intact, but more readily hydrolyzable than normal cellulose. 5. Hydrolysis at (2), (3), and (4) would rupture the corresponding rings to give a dextrin with reducing properties. It would be possible to obtain a dextrin without reducing properties by fission at (3) and (4), or at (5) and (6). The dextrins would be more or less readily hydrolyzed, since it is improbable that there is much stability to the facial 10 and 12-membered rings in themselves."

Gray[43] suggests a somewhat different formula for cellulose, based on four molecules of glucose. He notes that one hydroxyl group per each 24 carbon atoms shows a different reactivity from the other 11 free hydroxyl groups. Gray's formula accordingly consists of four glucose residues, three of which contain the δ-oxide ring and the fourth the γ-oxide ring. The hydroxyl group which is starred in the following formula should have different properties from the other hydroxyl groups.



X-ray studies have indicated that the cellulose fiber possesses an essentially crystalline structure, at least insofar as the regular spacing of the carbon, hydrogen, and oxygen atoms is concerned. Sponsler and

[43] Gray, H. LeB., A Suggested Constitutional Formula for Cellulose, *Ind. Eng Chem.*, 18 : 811 (1926).

Dore[44] have studied the structure of ramie cellulose and have prepared models of the ramie cellulose molecule and present photographs of such models in their paper. They conclude that the glucose units possess

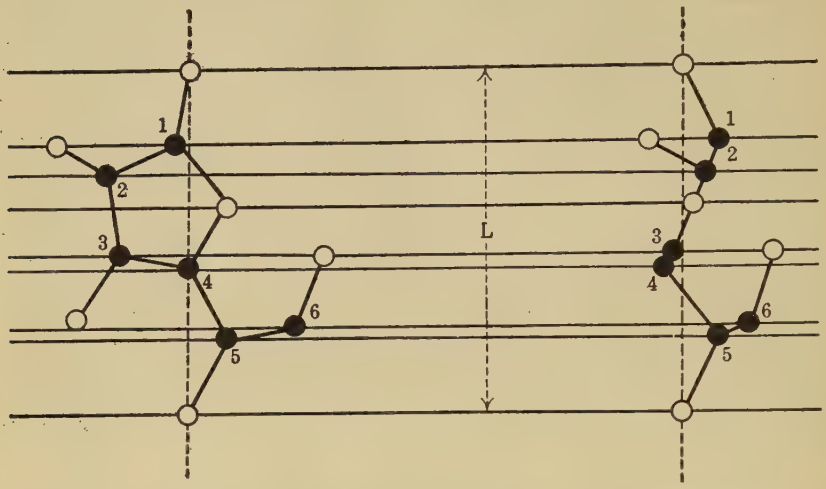


FIG. 121.—A skeleton drawing of the butylene oxide unit of glucose in cellulose, as viewed directly and at right angles to the plane of the unit, showing the space relations of the atoms which should appear in an X-ray diagram. (After Sponsler and Dore.)

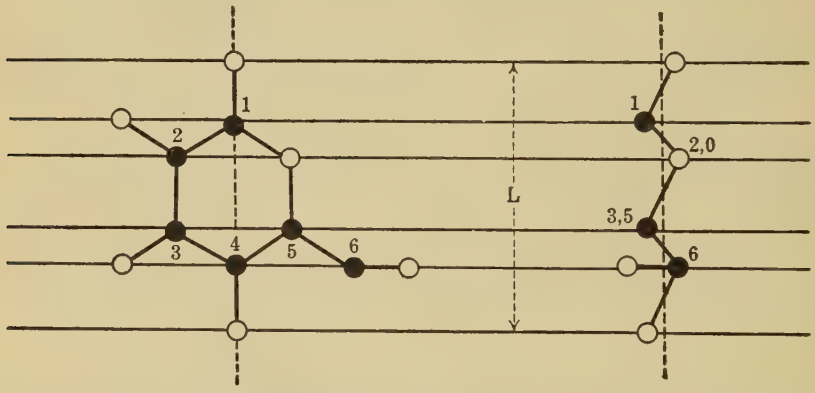


FIG. 122.—A skeleton drawing of the amylene oxide unit of glucose in cellulose, as viewed directly and at right angles to the plane of the unit, showing the space relations of the atoms which should appear in an X-ray diagram. This spacing agrees with experimental findings. (After Sponsler and Dore.)

[44] Sponsler, O. L., and Dore, W. H., The Structure of Ramie Cellulose as Derived from X-Ray Data, Colloid Symposium Monograph, Vol. 4, pp. 174–202, Chemical Catalog Company, Inc., New York (1926).

the δ-oxide ring rather than the γ-oxide ring, inasmuch as measurements of the distance (L) gave a spacing of 5.40 Å between butylene oxide units and 5.10 Å between amylene oxide units, and the X-ray photographs showed that the distance (L) in the ramie cellulose was 5.15 Å, very closely approximating the requirement for the amylene oxide form.

Figures 121 and 122, reproduced from the paper of Sponsler and Dore, show the spatial relationships of the carbon and oxygen atoms of the

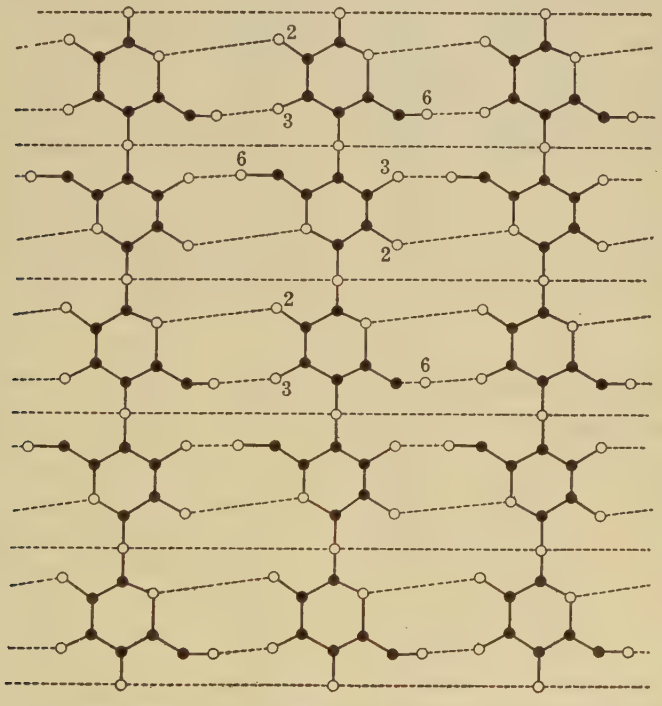


Fig. 123.—Structure of cellulose. Tangential section through a ramie fiber showing three chains of glucose units. Dark lines indicate primary valence bonds. Broken lines indicate probable general direction of secondary valence forces. (After Sponsler and Dore.)

butylene oxide and amylene oxide forms of glucose. The left-hand diagram of each figure shows the units as they would appear in a photograph. Those on the right represent the model at right angles to the photographed position.

Figure 123 is their diagrammatic representation of the arrangement of the glucose molecules in a tangential section through a ramie fiber. Figure 124 is a similar radial section through a ramie fiber. The black circles are carbon atoms, the open circles oxygen atoms; the solid lines

are bonds of primary valence, whereas the broken lines are the direction of secondary valence forces.

Sponsler and Dore conclude that the cellulose fiber is made up of chains of glucose molecules held together by the bonds of primary valence, these chains of molecules in turn being associated and held together by the bonds of secondary valence. They note that cellulose fibers, in general, show little or no swelling in a longitudinal direction, which could be accounted for because the linkages in the longitudinal direction of the fiber are the linkages of primary valence. Accordingly there is no opportunity for water molecules to penetrate between the glucose radicals, so that longitudinal swelling cannot occur to any great extent. On the other hand, cellulose fibers swell greatly in a lateral direction. Here the secondary valence forces are involved. The water molecules of the medium would become adsorbed upon the oxygen atoms, which would tend to increase the distance between the parallel chains of glucose molecules. They note that X-ray crystal structure observations bear out these assumptions. Similarly, the tensile strength studies have shown that the fiber is much stronger in a longitudinal direction than in a lateral direction.

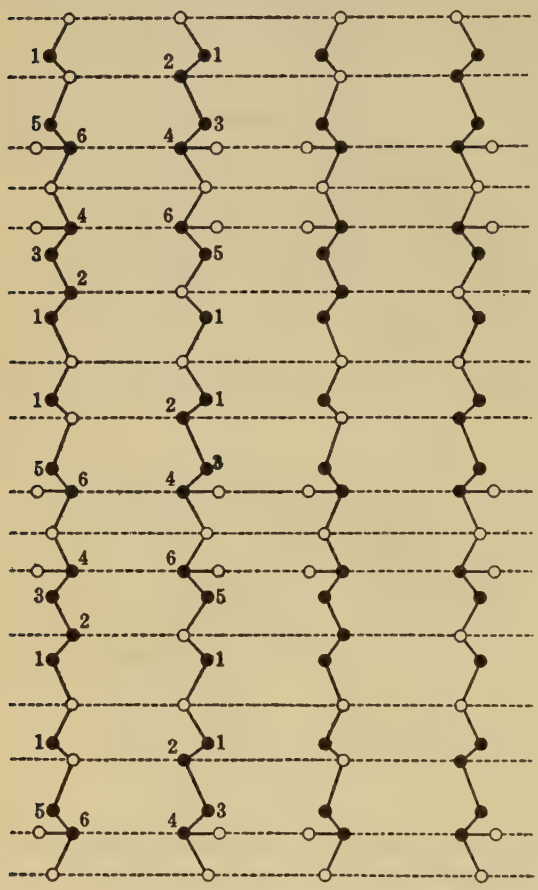


Fig. 124.—Structure of cellulose. A radial section through a ramie fiber showing primary and secondary valence bonds. (After Sponsler and Dore.)

Sponsler and Dore further note that a group of eight glucose

units is the simplest unit which can represent the structure of cellulose.

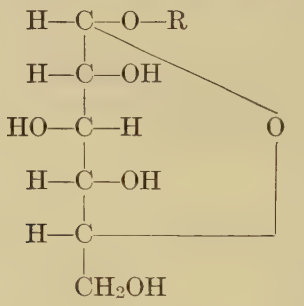
In a later paper, Sponsler and Dore[45] considered the change which is produced in the space lattice structure of ramie cellulose that has been "mercerized" by treatment with sodium hydroxide. They find that there are three processes which are involved: (1) a shift in the position of the chain of glucose residues with respect to the neighboring chains, (2) a partial rotation of the alternate glucose units in each chain, and (3) a shift in the spatial relationships of the hydroxyl group which is attached to the sixth carbon atom. They find that no mercerization takes place in alkali less concentrated than 13 per cent and state that it is extremely unlikely that the mercerization process involves either the migration of an oxygen bridge, enolization, or a molecular rearrangement of the glucose units.

[45] Sponsler, O. L., and Dore, W. H., The Structure of Mercerized Cellulose. I. The Space Lattice of Mercerized Ramie Cellulose as Developed from X-Ray Data, *J. Am. Chem. Soc.*, 50 : 1940–1950 (1928).

CHAPTER XXVI

THE GLUCOSIDES

The synthetic glucosides and the naturally-occurring glucosides are similar in structure, and the formula may be represented by,



where R represents the foreign (non-sugar) group. This may be coniferyl alcohol as in the glucoside, *coniferin*, or salicyl alcohol as in *salicin*, or aniline, or benzaldehyde, etc., as in various glucosides.

The α- and β-forms of the glucosides have already been discussed. The methyl and ethyl glucosides and galactosides and the methyl xylosides are known in both α- and β-forms, but *all naturally-occurring glucosides, so far as is known, occur only in the β-form* and are hydrolyzed only by emulsin.

The rotatory power of an unknown glucoside (*e.g.*, the α-form) can be calculated from the optical rotation of the known β-form because

$$\text{Rotation of } \frac{\alpha + \beta \text{ glucosides}}{2} = \text{rotation of } \frac{\alpha + \beta \text{ glucose}}{2}$$

when R is not optically active (cf. Hudson[1]).

The role of the glucosides in the plants has been interpreted as a mechanism whereby the substances which have great physiological activity are held inert until they are needed in the metabolism of the

[1] Hudson, C. S., The Significance of Certain Numerical Relations in the Sugar Group, *J. Am. Chem. Soc.*, 31 : 66–86 (1909).

plant or in rendering poisonous substances inert so that they will not injure the plant cells (cf. Combes,[2, 3] and Clark[4]).

The glucosides are widely distributed in nature but usually occur in small amounts. The non-sugar residue is in most instances an aromatic compound. Only sugars capable of forming a δ-oxide ring form glucosides in nature, and most of the naturally-occurring glucosides are compounds of glucose, although arabinose, xylose, ribose, rhamnose, galactose, mannose, and fructose have been isolated from glucosides.[5]

The glucosides are most commonly found in the fruit, bark, or roots of plants, although they frequently occur in the leaves. They are usually levo-rotatory, crystalline, colorless, bitter, soluble in water or in alcohol. In order to isolate them without hydrolysis, it is desirable to destroy their accompanying enzyme by heat. They are easily hydrolyzed by emulsin or by dilute mineral acids.

The glucosides may be classified according to the nature of the non-sugar part of the molecule, under phenols, alcohols, aldehydes, acids, etc.

A list of a few of the more important of the naturally-occurring glucosides appears on pages 572 and 573.

Certain glucosides contain two hexose molecules, *e.g.*, amygdalin which contains 2 glucose + benzaldehyde + HCN (as the nitrile). By appropriate technic one carbohydrate group can in some instances be hydrolyzed off, yielding a new glucoside containing only *one* hexose.

Both on account of the very small amount of glucosides present in plant tissue and the fact that, as a rule, glucosides do not form characteristic insoluble derivatives which allow for their isolation and identification, it is difficult to discover new glucosides and still more difficult to determine their constitution. Certain biochemical methods have assisted materially in this work:

1. *Bourquelot's method* for examining a plant for glucosides consists in determining the reducing sugar and the optical rotation of an extract of the plant tissue. Then he adds emulsin and after a period of incubation redetermines the reducing sugars and the optical rotation. An

[2] Combes, R., Recherches biochimiques expérimentales sur le rôle physiologique des glucosides chez les végétaux, *Rev. gen. botan.*, Vols. 29 and 30 (1917–18). This article, including a total of 224 pages begins on p. 321 of Vol. 29 and is continued in 13 instalments, ending on p. 363 of Vol. 30.

[3] Combes, R., Immunité des végétaux vis-à-vis des principes immédiats qu'ils élaborent, *Compt. rend.*, 167 : 275–278 (1918).

[4] Clark, E. D., Note on the Blackening of the Leaves of the Wild Indigo (*Baptisia tinctoria*) and the Isolation of a New Phenol, Baptisol, *J. Biol. Chem.*, 21 : 645–660 (1915).

[5] The term, glucoside, refers to a *type* of compound; glucose is not necessarily a component.

Name	Hydrolytic Products	Source
	I. Phenols	
Arbutin	Glucose + hydroquinone	*Arbutus uva ursi*
Phloridzin	Glucose + phloretin	Bark of *Rosaceae*
	II. Alcohols	
Coniferin	Glucose + coniferyl alcohol	Bark of fir tree
Populin	Glucose + saligenin + benzoic acid	Bark of *Populus*
Salicin	Glucose + saligenin	Bark of willow (*Salix* sp.)
	III. Aldehydes	
Amygdalin	2 Glucose + *d*-mandelonitrile	Seeds of bitter almond
Dhurrin	Glucose + *p*-oxymandelonitrile	Leaves of *Sorghum vulgare*
Linamarin	Glucose + acetonecyanhydrin	Leaves of young flax plants and flax seed
Prulaurasin	Glucose + racemic mandelonitrile	Leaves of *Prunus laurocerasus*
Prunasin	Glucose + *d*-mandelonitrile	Young twigs of *Prunus padus*
Sambunigrin	Glucose + *l*-mandelonitrile	Leaves of common elder (*Sambucus niger*)
	IV. Acids	
Gaultherin	Glucose + methylsalicylate	Bark of *Betula lenta* or *Gaultheria procumbens*
Jalapin	Glucose + jalapinolic acid	Roots of *Jalapa orizabenzis*
	V. Oxycumarin Derivatives	
Aesculin	Glucose + aesculetin	Bark of horse-chestnut (*Aesculus hippocastanum*)
Fraxin	Glucose + fraxetin	Bark of the ash (*Fraxinus* sp.)
	VI. Oxyanthraquinone Derivatives	
Ruberythric acid	Glucose + alizarin	Madder, the roots of *Rubia tinctorum*

Name	Hydrolytic Products	Source
	VII. Oxyflavone Derivatives	
Apiin	Apiose + apigenin	Leaves of parsley, celery, etc.
Quercitrin	Rhamnose + quercetin	Bark of oak
Rutin	Glucose + rhamnose + quercetin	Petals of the violet, leaves of *Ruta graveolens*, etc.
Xanthorhamnin	2 Rhamnose + galactose + rhamnetin	Fruits of various species of *Rhamnus*
	VIII. Mustard Oils	
Sinigrin	Glucose + allyl isothiocyanate + $KHSO_4$	Black mustard seeds
	IX. Anthocyans	
Cyanin	2 Glucose + cyanidin	Flowers of cornflower, rose, etc.
Delphinin	2 Glucose + 2 *p*-oxybenzoic acid + delphinidin	Flowers of larkspur
Oenin	Glucose + oenidin	Skins of purple grapes
Pelargonin	2 Glucose + pelargonidin	Flowers of geranium
	X. Digitalis Group	
Digitalin	Glucose + digitalose ($C_7H_{14}O_5$) + digitaligenin	Leaves of foxglove
Digitonin	2 Glucose + 2 galactose + digitogenin	Leaves of foxglove
Digitoxin	2 Digitoxose ($C_6H_{12}O_4$) + digitoxigenin	Leaves of foxglove
	XI. Sapogenins	
Phytosterolin	Glucose + a sitosterol	Sarsaparilla, root of *Smilax* sp.
Sarsasaponin	3 Glucose + sarsasapogenin, $C_{26}H_{41}O_2(OH)$	Sarsaparilla, root of *Smilax* sp.
	XII. Various	
Dibenzoylglucoxylose	Glucoxylose + benzoic acid	Leaves of *Daviesia latifolia*
Indican	Glucose + indoxyl	Leaves of *Indigofera*

increase in optical rotation indicates the presence of β-glucosides, and the amount of change may give a rough estimation of the amounts which are present.

2. The nature of the sugar can be more or less accurately determined, according to ter Meulen, by adding the different sugars to the glucoside + enzyme mixture. The sugar which retards the enzyme action is probably the one in the glucoside, inasmuch as the hydrolysis is an equilibrium reaction.

$$\text{Glucoside} + \text{enzyme} + H_2O \rightleftharpoons \text{enzyme} + \text{sugar} + \text{non-sugar residue.}$$

This consideration possibly led to Bourquelot's *enzyme synthesis* work which established definitely that enzyme actions were equilibria and were reversible. The first demonstration of synthesis by enzyme action was carried out in the case of glucosides. Emulsin was added to a concentrated solution of the split products of the glucoside, and the mixture was incubated at 37° C. It was found that the equilibrium,

$$\text{Glucoside} + H_2O \rightleftharpoons \text{sugar} + \text{non-sugar residue,}$$

was shifted toward the left, *i.e., the amount of reducing sugar decreased.* The synthesis of glucosides was thus definitely proven. Upon dilution of the system, the reaction again shifted to the right, and hydrolysis of the glucoside took place.

Bridel and Béguin[6] have recently used this method of enzyme synthesis for the preparation of ethyl *l*-arabinose by adding emulsin to a solution of pure *l*-arabinose in alcohol and allowing the mixture to stand for 173 days, adding more emulsin on the 27th and 43rd days. They state that the compound isolated was α-ethyl *l*-arabinoside and suggest that possibly the commercial emulsin contained as a specific enzyme, α-*l*-arabinosidase.

Cyanogenetic or Cyanophoric Glucosides.—These glucosides have received a considerable amount of attention not only because of their practical importance, being present in such substances as bitter almonds, linseed cake, sorghum, and other fodder plants, etc., but also because of theoretical interest as to their function in plants. They contain nitrogen as HCN, and because of that fact can be more or less easily estimated. HCN is fairly common among higher plants and occurs, in the majority of cases, combined in glucosides.

Although the cyanogenetic glucosides are somewhat rare as compared with saponins, they have a much wider distribution than was once supposed, and are present in many economic plants. Flax contains a

[6] Bridel, M., and Béguin, C., Synthèse biochimique, à l'aide de l'émulsine des amandes, de l'ethyl-*l*-arabinoside α, *Compt. rend.*, 182 : 812–814 (1926).

small amount of HCN in the dry seed (0.008 per cent HCN), but as much as 0.135 per cent has been found in the germinated seedlings. The same increase occurs in the germination of bitter almonds. The stage of development at which the maximum amount is reached may be different for different plants. Thus, in flax the maximum occurs when the seedlings are 4 to 5 inches high, while in *Lotus arabicus* the maximum is at the time of flowering. In sorghum there appears to be no HCN in the seed, but Willaman found the maximum amount in seedlings about 3 inches high, decreasing to practically zero in the mature plant. During the first three weeks the HCN was concentrated in the stalks, then passed largely to the leaves, and persisted there more or less until maturity. The temperature and soil condition play a major part in determining the amount of HCN in sorghum; cold, wet days in the early growing period markedly increase the HCN content and cold days in the fall cause the reappearance of HCN. This is particularly true of " suckers " in a sorghum field just before or after frost. Frost naturally destroys protoplasmic structure and permits the enzyme to hydrolyze the glucoside, so that cattle are readily poisoned in a sorghum field following a frost. Horses are much more resistant to the toxic action of HCN.

Uncombined HCN has been found in plants in a few instances, but it is extremely rare, and the presence of HCN may be considered as evidence of the presence of a cyanophoric glucoside. Therefore, the quantitative determination of the cyanophoric glucosides consists in the determination of HCN, formed by the hydrolysis of the glucosides, (a) by autolyzing the plant tissue, or (b) by addition of acid, or (c) by incubating, following the addition of emulsin, and distilling off the HCN into alkali. Autolysis was the first method employed, in fact the autolysis of crushed bitter almonds was the first means by which HCN was identified as a plant constituent.

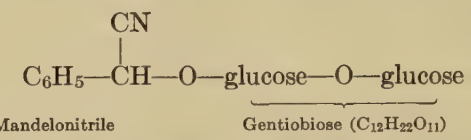
A great deal of attention has been given to methods for the determination of HCN, and the various factors governing its quantitative liberation, quantitative distillation, and measurement have been exhaustively studied. There is still much room for improvement in the present methods. In most cases one deals with quantities ranging from a fraction of a milligram to one or two milligrams of HCN, and the measurement depends upon the conversion of ferrous salts to Prussian blue. The conditions of formation must be such that the pigment will remain suspended in a colloidal sol, so that the measurement can be made colorimetrically (cf. Willaman[7]).

Amygdalin, $C_{20}H_{27}O_{11}N$, is probably the best known of the glu-

[7] Willaman, J. J., The Estimation of Hydrocyanic Acid and the Probable Form in Which It Occurs in *Sorghum vulgare*, *J. Biol. Chem.*, 29 : 25–36 (1917).

cosides. It is obtained from the kernels of the bitter almond, *Prunus amygdalus*. It also occurs in smaller amounts in the kernels of the peach, plum, apricot, and most fruits belonging to the *Rosaceae*.

Liebig and Wöhler, in 1837, found that amygdalin was hydrolyzed to glucose, benzaldehyde and HCN. Caldwell and Courtauld showed that the reaction took place in two stages when acids were used as the hydrolyzing agents, one glucose radical being split off before the second one was attacked. The structure has been shown to be,



Emulsin hydrolyzes the two glucose bonds at about the same rate, so that the end products of emulsin hydrolysis are two molecules of glucose and mandelonitrile which further hydrolyzes into benzaldehyde and HCN. Amygdalase hydrolyzes off only one glucose radical, forming a new glucoside, *prunasin*, which in turn is hydrolyzed by the enzyme, *prunase*. The enzyme, prunase, was first found in the leaves of the cherry laurel, hence the name. "Emulsin" therefore contains two enzymes, amygdalase and prunase. Prunase cannot act until the amygdalase has split off one glucose radical. This is another example of enzyme biological specificity and indicates that one glucose radical shields the rest of the molecule from the enzyme.

Three glucosides of the same formula and composition as prunasin are known:

1. Prunasin *d*-mandelonitrile glucoside
2. Prulaurasin *d-l*-(racemic) mandelonitrile glucoside
3. Sambunigrin *l*-mandelonitrile glucoside

This is one of the few instances where the *d*, *l*, and racemic forms of a compound all occur in nature.

Dhurrin occurs in *Sorghum vulgare* and was isolated by Dustan and Henry in 1902. Its structure is *p*-hydroxymandelonitrile glucoside, hydrolyzing to glucose, HCN, and *p*-hydroxybenzaldehyde. It also occurs in the forage crop, "Sudan grass." This glucoside is probably responsible for the death of many cattle feeding in sorghum and Sudan grass fields in the autumn.

Willaman[8-10] believes that HCN exists in two forms in sorghum,

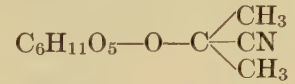
[8] Willaman, J. J., The Effect of Anesthetics and of Frosting on the Cyanogenetic Compounds of *Sorghum vulgare*, *J. Biol. Chem.*, 29 : 37–45 (1917).

[9] Willaman, J. J., and West, R. M., Notes on the Hydrocyanic Acid Content of Sorghum, *J. Agr. Res.*, 4 : 179–185 (1915).

(1) as dhurrin, and (2) in a loosely combined form, perhaps as free HCN, but in any event as a non-glucosidic form. In his study he made some interesting observations on the effect of anaesthetics on plants. Certain bulbs, etc., can be " forced " by anaesthetics so that they will bloom out of season. This has been believed to be due to an altered permeability of the cells, allowing for a more rapid absorption of water and allowing for the bringing together of enzyme and substrate. Willaman found that when sorghum leaves were treated with chloroform, ether, or alcohol, more HCN, both glucosidic and non-glucosidic, was obtained than was secured from normal leaves. He believes that the anaesthetic stimulated the hydrolytic processes so that a part of the dhurrin already present was hydrolyzed, but that the *synthetic* processes were also stimulated and more *dhurrin and HCN were also synthesized.* This appears to be one of the few instances where the synthetic action of a plant or of plant cells can be stimulated by chemical manipulation. It does not prove, however, that the same enzyme causes both the hydrolysis and the synthesis, in fact it points strongly to the opposite conclucion, *i.e.*, that we are " activating " two enzymes which work in opposing directions, one toward synthesis and the other toward hydrolysis.

Vicianin, $C_{19}H_{25}O_{10}N$, occurs in the seeds of the wild vetch, *Vicia angustiafolia*, and yields on hydrolysis HCN, benzaldehyde, and a disaccharide, vicianose (glucose + arabinose). It may be noted that this glucoside is similar to amygdalin except that one glucose radical of amygdalin has been replaced by the pentose, arabinose. The wild vetch seed contains a relatively small amount of glucoside (HCN = 0.0033 per cent of the seed), but a small amount may produce undesirable effects. The vetch is one of the so-called " non-separable " weed seeds in grain, and the presence of vetch in wheat will produce a pronounced benzaldehyde odor in bread doughs made from the flour.

Linamarin or *phaseolunatin*,



hydrolyzes to β-glucose, acetone, and HCN. It occurs in flaxseed, flax plants, etc. (cf. Collins and Blair[11]). Henry and Auld[12] pointed out that flaxseed meal was non-toxic when used as an animal feed, because the

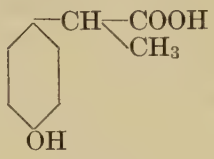
[10] Willaman, J. J., and West, R. M., Effect of Climatic Factors on the Hydrocyanic Acid Content of Sorghum, *J. Agr. Res.*, 6 : 261–272 (1916).

[11] Collins, S. H., and Blair, H., Rate of Liberation of Hydrocyanic Acid from Commercial Kinds of Linseed, *Chem. News*, 111 : 19–20 (1915).

[12] Henry, T. A., and Auld, S. J. M., Occurrence of Cyanogenetic Glucosides in Feeding Stuffs, *J. Soc. Chem. Ind.*, 27 : 428–433 (1908).

enzyme which brings about hydrolysis had been destroyed by the heat used in pressing out the oil. Almy and Robinson,[13] however, find that flaxseed meal is very toxic when fed to trout. In some way it undergoes hydrolysis in their digestive tract, and they are poisoned by the HCN.

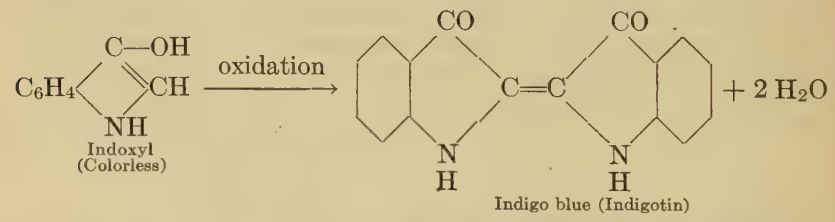
Phloridzin, yielding, as the products of acid hydrolysis, glucose, phloroglucinol, and *p*-oxyhydratropic acid,



is found in the bark of the apple, pear, and other rosaceous trees. It is not hydrolyzed by emulsin. Phloridzin possesses the remarkable property of causing " artificial diabetes " or glycosuria when taken internally, or better when injected subcutaneously (in oil). Much of the experimental work on diabetes has been suggested by experiments made on phloridzinized dogs.

Salicin, a glucoside hydrolyzing to β-glucose and saligenin (*o*-hydroxybenzyl alcohol) occurs in the bark of most species of *Salix* (willows). This glucoside is assuming increased importance because of the action of saligenin as a local anaesthetic. Kunz[14] has recently described a new synthesis of salicin and has reported a study of the abnormal optical rotations of certain salicin derivatives.

Indican, $C_{14}H_{17}O_6N$, on hydrolysis yields glucose and indoxyl. Indoxyl is the leuco-base of indigo blue to which it passes on oxidation.



The glucoside occurs in the indigo plants and can be extracted by acetone. A specific enzyme is present in the leaf which hydrolyzes the glucoside, and the indoxyl in the presence of air and of an *oxidase* is converted to the indigo blue. The sugar radical must be split off before

[13] Almy, L. H., and Robinson, R. K., Toxic Action of Ingested Linseed Meal on Trout, *J. Biol. Chem.*, 43 : 97–111 (1920).

[14] Kunz, A., Studies on Salicin. I. Exceptional Rotations of the Halogeno-tetra-acetyl Derivatives of Salicin. A New Synthesis of Salicin, *J. Am. Chem. Soc.*, 48 : 262–268 (1926).

oxidation can take place. This is one of the best known cases where one enzyme (glucosidase) must act to produce a compound before a second enzyme (oxidase) can act to produce a third substance.

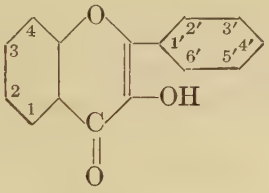
The production of indigo from indican was at one time one of the world's great industries, and the indigo planters of India were practically ruined by Baeyer's synthesis of indigo. In carrying out the successful synthesis, the Badische Company spent nearly $5,000,000 during seventeen years of research before a pound of synthetic indigo had been sold.

At the present time natural indigo is not a serious competitor of synthetic indigo except in the orient where cheap labor is available and an inferior product is acceptable. The crude natural indigo often contains only 5 to 7 per cent of dyestuff.

Although indigo occurs exclusively as a glucoside in the plant kingdom, and in the animal body as a derivative of tryptophane formed by bacterial action, a brom-substituted indigo occurs in a gland in the molluscs, *Murex brandaris* and *Murex trunculus*. This compound, 6.6′-dibrom indigo, was known to the ancients as " Tyrian purple." Friedländer[15, 16] isolated 1.5 grams of the dyestuff from 12,000 molluscs. Research has shown that the synthetic 5.5′-dibrom indigo dyes cloth a clearer purple and is much to be preferred to the " Tyrian purple " as a dyestuff. This is again an instance where the chemist in his laboratory has prepared a product superior, for industrial purposes, to those found in nature.

6.6′-Dibrom indigo is one of the few naturally-occurring organic compounds which contain bromine.

The hydroxyflavone glucosides contain flavonol, or hydroxyflavone, or some derivative of this, as the non-sugar radical.



Flavonol

All of the flavonol derivatives are yellow dyes. In many of these glucosides the carbohydrate is rhamnose. Quercitrin occurs in oak bark. On hydrolysis it yields quercetin (1.3.3′4′-tetrahydroxyflavonol)

[15] Friedländer, P., Ueber schwefelhaltige Analoga der Indigogruppe, *Ber.*, 39 : 1060–1066 (1906).

[16] Friedländer, P., Über den Farbstoff des antiken Purpurs aus Murex brandaris, *Ber.*, 42 : 765–770 (1909).

and rhamnose. Such glucosides are usually the raw materials used for the preparation of rhamnose (cf. Walton[17]). Quercetin is widespread as a plant pigment, occurring in red onion skins, cotton flower, etc.

Saponins.—The saponins comprise a large group of compounds widely distributed in the higher plants. They are mostly amorphous substances, soluble in water to colloidal solutions which foam readily and which stabilize emulsions of fats and oils, so that such emulsions are very difficult to "break." The name signifies that they are " soap-like." They greatly reduce surface tension. The " soap weeds," " soap bushes," etc., all owe their detergent effect to saponins. In certain instances they have been used to furnish foam on manufactured beverages. This is, however, now generally prohibited by law because of the undesirable physiological action of the saponins.

On hydrolysis with mineral acids they yield sugars, such as glucose, galactose, arabinose, and rhamnose, together with " sapogenins," more or less physiologically active nuclei of unknown and complex structure.

The most important group of saponins, from the medical standpoint, is that of the foxglove, *Digitalis purpurea*. This plant contains at least five glucosides which are the active principles of the digitalis extract used so often in diseases of the heart, decreasing the frequency and strengthening the intensity of the beat. No artificial or synthetic drug has been found to replace digitalis in medicine.

Digitonin, which forms approximately one-half of the glucosides of digitalis, is a saponin hydrolyzable to two molecules of glucose, two molecules of galactose, and digitogenin.

$$\underset{\text{Digitonin}}{C_{54}H_{92}O_{28}} + 2H_2O \rightarrow \underset{\text{Glucose}}{2C_6H_{12}O_6} + \underset{\text{Galactose}}{2C_6H_{12}O_6} + \underset{\text{Digitogenin}}{C_{30}H_{48}O_6}$$

Digitonin is likewise used in the quantitative estimation of cholesterol and phytosterol with which it forms an insoluble compound (cf. Windaus,[18] Windaus and Shah,[19] and Palmer and Eckles[20]). The digitonin may later be recovered from the cholesterol-digitonin precipitate by the method of Panzer.[21]

[17] Walton, C. F., Jr., The Preparation of Rhamnose, *J. Am. Chem. Soc.*, 43 : 127–131 (1921).

[18] Windaus, A., Über die quantitative Bestimmung des Cholesterins und der Cholesterinester in einigen normalen und pathologischen Nieren, *Z. physiol. Chem.*, 65 : 110–117 (1910).

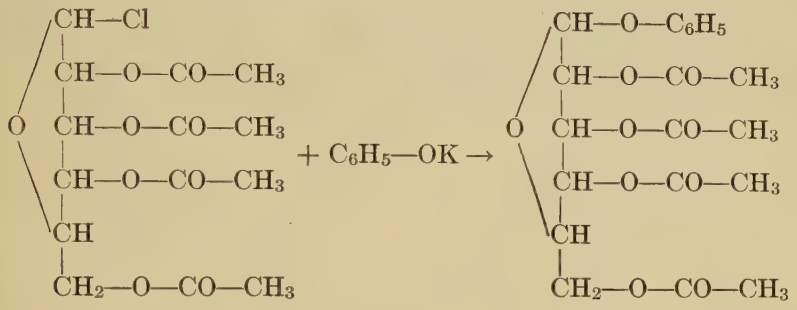
[19] Windaus, A., and Shah, S. V., Über einige neue Abbauprodukte des Digitogenins, *Z. physiol. Chem.*, 151 : 86–97 (1926).

[20] Palmer, L. S., and Eckles, C. H., Carotin—The Principal Natural Yellow Pigment of Milk Fat—Pt. II, *Res. Bull.* No. 10, Missouri Agr. Exp. Sta. (1914).

[21] Panzer, T., Notizen über die chemische Zusammensetzung der Tuberkelbazillen, *Z. physiol. Chem.*, 78 : 414–419 (1912).

All of the saponins are characterized by marked physiological action and toxic properties. Fish, in particular, are extremely sensitive, being killed by 1 : 100,000 solutions. Most of the fish poisons of the aborigines are saponins. They exhibit a solvent action on red corpuscles, causing hemolysis. The hemolytic effect is possibly due to a withdrawal of cholesterol from the corpuscle wall.

Synthesis of Glucosides.—If we add the sodium or potassium salt of a phenol to acetobromglucose or acetochlorglucose (from a pentaacetyl sugar treated with anhydrous HCl or HBr, cf. Fischer [22]), we obtain the corresponding phenol derivative.



The acetyl groups are then removed by hydrolyzing with dilute alkali, resulting in the formation of the glucoside. A number of the simpler natural glucosides have been synthesized by the above or similar methods.

Fischer synthesized certain of the α-series and found that none of these were hydrolyzed by emulsin. He also prepared glucosides, galactosides, and rhamnosides of the purines, *e.g.*, adenine, guanine, xanthine, hypoxanthine, and theobromine (cf. Fischer[23-25]). In this instance the compounds which are formed may be somewhat different in structure from the naturally-occurring glucosides, which have already been considered, in that the linkage from the sugar to the non-sugar may not be through an oxygen but rather may be a direct bond from carbon to nitrogen.

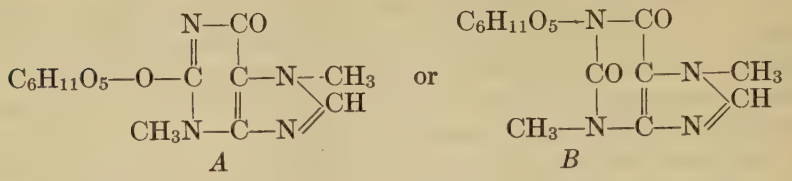
[22] Fischer, E., and Armstrong, E. F., Über die isomeren Acetohalogen-Derivate des Traubenzuckers und die Synthese der Glucoside, I–III, *Ber.*, 34 : 2885–2900 (1901); 35 : 833–843 (1902); 35 : 3153–3155 (1902).

[23] Fischer, E., and Helferich, B., Synthetische Glucoside der Purine, *Ber.*, 47 : 210–235 (1914).

[24] Fischer, E., and Fodor, K. v., Notiz über Theophyllin-rhamnosid, *Ber.*, 47 : 1058–1061 (1914).

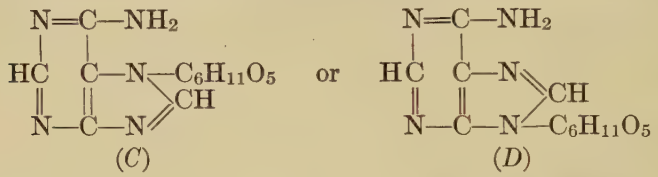
[25] Helferich, B., and Kühlewein, M. v., Synthese einiger Purin-glucoside, *Ber.*, 53 : 17–22 (1920).

Thus, for theobromine we have the possibilities of either



In (*A*) we have a true glucosidal linkage through oxygen, whereas in (*B*) the union is from carbon to nitrogen. Fischer was unable to decide which of these formulae represented the compound which was isolated.

In compounds where the purine or pyrimidine does not contain oxygen, *e.g.*, adenine, there is no possibility of the true glucosidal linkage. In this instance, Fischer presents the alternative formulae,



and notes that it was impossible to distinguish with certainty between these formulae, although he was inclined to regard formula (*C*) as the more probable. Such compounds are probably very closely related to the nucleosides derived from nucleic acid, except that *d*-ribose is the sugar present in the nucleosides. As a matter of fact Fischer's[26] phosphoric acid esters of the purine glucosides may be regarded as synthetic nucleotides.

Asymmetric Synthesis.—Fischer suggests that the optical activity of the sugar molecule may be responsible for the formation in nature of various optically active components on the hypothesis that the optically active compounds are perhaps originally associated with a sugar molecule and later are hydrolyzed off. Fischer and Slimmer[27] have presented certain proof for this hypothesis. Thus, using the glucoside, *helicin*, in which the non-sugar radical is salicylic aldehyde, they found that it was possible to prepare the cyanhydrin by the addition of HCN which could be later hydrolyzed to the corresponding acid amide. On further hydrolysis the sugar molecule was split off, yielding an optically active orthohydroxymandelic acid,

[26] Fischer, E., Über Phosphorsäureester des Methyl-glucosids und Theophyllin-glucosids, *Ber.*, 47 : 3193–3205 (1914).

[27] Fischer, E., and Slimmer, M., Versuche über asymmetrische Synthese, *Ber.*, 36 : 2575–2587 (1903).

$$C_6H_{11}O_5—O—C_6H_4CHO \xrightarrow{HCN}$$

$$C_6H_{11}O_5—O—C_6H_4—\underset{H}{\overset{CN}{\underset{|}{\overset{|}{C}}}}—OH \xrightarrow{\text{hydrolysis}}$$

$$C_6H_{11}O_5—O—C_6H_4—\underset{H}{\overset{OH}{\underset{|}{\overset{|}{C}}}}—CO—NH_2 \xrightarrow{\text{hydrolysis}}$$

$$C_6H_{12}O_6 + OH—C_6H_4—\underset{H}{\overset{OH}{\underset{|}{\overset{|}{C}}}}—COOH$$

o-Hydroxymandelic acid
(Optically active)

In a somewhat analogous manner, the same authors prepared ortho-hydroxyphenylethyl carbinol,

$$OH—C_6H_4—\underset{H}{\overset{OH}{\underset{|}{\overset{|}{C}}}}—C_2H_5$$

which again showed a relatively high specific optical rotation, $-9.83°$.

Fischer notes that he could obtain no evidence for the formation of more than one of the optically active isomers, and in these instances at least there is no possibility that α- and β-forms were originally present to account for the asymmetric synthesis, inasmuch as the glucose radical was originally present entirely in the β-form and in itself did not enter into the synthetic reactions, although it must have influenced them.

CHAPTER XXVII

THE PECTIC SUBSTANCES*

The term, " pectin," is used to denote the substance or substances which, in the presence of the proper concentrations of acid and sucrose, will form the familiar fruit jellies and jams. The pectin producing these jellies is the only water-soluble member of a group of related compounds known as the " pectic substances," or sometimes called the " pectins." At the present time there are generally recognized three of these substances, *protopectin*, *pectin*, and *pectic acid*. There is some evidence for recognizing two others, *pectose* and *pectinic acids*.

The pectic substances are colloidal carbohydrates of high molecular weight and rather complex composition. Galacturonic acid, galactose, arabinose, methanol, and acetic acid have been identified as hydrolytic products. However, as the presence of both galactose and arabinose has been questioned, a brief summary of the evidence for each substance may be desirable.

Galacturonic acid was isolated by Ehrlich[1] from beet pectin after hydrolysis of the latter with oxalic acid. As already indicated in the discussion of the uronic acids, it is probably formed in the plant by oxidation of the primary alcohol group of galactose. On treatment with nitric acid, the galacturonic acid is oxidized to mucic acid. It has long been known that mucic acid is one of the products which can be obtained from pectin treated with nitric acid. Arabinose is formed by decarboxylation of the galacturonic acid. Decarboxylation may be accomplished by boiling with 12 per cent hydrochloric acid,[2, 3] or less readily by heating

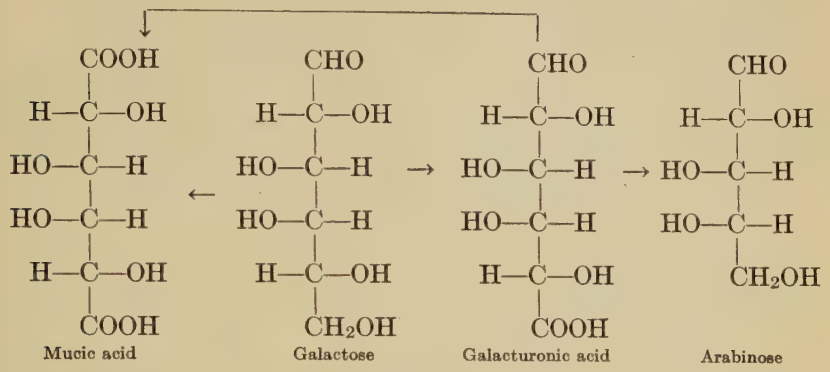
* This chapter is contributed by Dr. J. J. Willaman, Chief of the Division of Chemistry, of the New York Agricultural Experiment Station (Geneva), formerly Professor of Agricultural Biochemistry, in the University of Minnesota.

[1] Ehrlich, F., Die Pektinstoffe, ihre Konstitution und Bedeutung, *Chem. Ztg.*, 41 : 197–200 (1917).

[2] Lefèvre, K. U., and Tollens, B., Untersuchungen über die Glucuronsäure, ihre quantitative Bestimmung und ihre Farbenreaktionen, *Ber.*, 40 : 4513–4523 (1913).

[3] Nanji, D R., Paton, F. J., and Ling, A. R., Decarboxylation of Polysaccharide Acids; Its Application to the Establishment of the Constitution of Pectins and to Their Determination, *J. Soc. Chem. Ind.*, 44 : 253–258T (1925).

with alkali.[4] The interrelations of these substances are shown in the following formulae:



The formation of carbon dioxide when pectins were heated with acid indicated the presence of uronic acids long before Ehrlich isolated galacturonic acid. We now know that the latter occurs to the extent of 70 to 80 per cent in the various pectic substances.

Galactose has been recognized for a long time as a constituent of the pectins. Its presence was at first merely surmised from the fact that mucic acid is formed on treatment of pectins with nitric acid. Since, however, galacturonic acid also yields mucic acid, the isolation of the sugar from the hydrolytic products of the pectin furnished the necessary proof. It is unlikely that it is produced from any other substance during hydrolysis by weak acids.

On the other hand, there is real reason to question the presence of arabinose. It was at first surmised from the fact that the pectic substances give very strong reactions for pentoses; in fact, by the phloroglucide method of pentose estimation, a very high percentage of pentose in the pectin can be demonstrated. However, in this method furfural is formed by boiling the material with 12 per cent hydrochloric acid. This treatment will convert the galacturonic acid to arabinose, and then the latter to furfural. Hence, this phloroglucide method is not necessarily either a qualitative or a quantitative indication of pentose. Furthermore, although arabinose has been identified as such in the acid hydrolysate of pectins, it could easily be an artifact, due to decarboxylation of some of the galacturonic acid.

Methanol is undoubtedly present in pectin, and possibly in protopec-

[4] Candlin, E. J., and Schryver, S. B., Investigations of the Cell-Wall Substances of Plants, with Special Reference to the Chemical Changes Taking Place During Lignification, *Proc. Roy. Soc.*, 103B : 365–376 (1928).

tin. Fellenberg[5] showed that it is combined with pectin in ester linkages, and can be easily removed by saponification. Later work makes it seem probable that it is esterified with the carboxyl groups of the galacturonic acids. It has been identified beyond reasonable doubt by several methods.

Acetic acid has been definitely identified in the pectin of flax and of sugar beet by Ehrlich[6, 7] and in the sugar beet by Nelson.[8] Nelson could not find it in the pectin from the fruit of apple, tomato, or lemon.

Constitution of the Pectic Substances.—With the above brief enumeration of the constituent groups found in the pectins, we are in a position to discuss how these groups are combined in the various individual pectic substances.

Protopectin, as its name implies, is the mother substance of this group. It occurs in the cell walls of most plant tissues. The older name for this substance was pectose.

There are two places in plant cell walls where pectic compounds exist. Pectin compounds form the middle lamella, where they act as a cementing material between cells, and they likewise occur as incrustations or thickenings on the cell wall. There has been considerable shifting of opinion concerning the nature of the pectin compounds in these two regions. In the early history of pectin chemistry their identity was kept separate, and the existence of still other pectic compounds was hypothecated. Later it was thought simpler to consider all insoluble, cell-wall pectin as pectose. Still later this name was changed to protopectin. And at the present time there is very good evidence adduced to show that the cell-wall thickenings and the middle lamella are quite different in nature.[9, 10] Carré and Haynes call the material forming the thickenings, " pectose," and the other, "middle lamella pectin." Which of these should be called " protopectin," as the precursor of pectin, cannot be decided with our present knowledge. There is much evidence

[5] Fellenberg, Th. von, Über die Konstitution der Pektinkörper, *Biochem. Z.*, 85 : 118–161 (1918).

[6] Ehrlich, F., and Sommerfeld, R. von, Die Zusammensetzung der Pektinstoffe der Zuckerrübe, *Biochem. Z.*, 168 : 263–323 (1926).

[7] Ehrlich, F., and Schubert, F., Über die Chemie der Inkrusten des Flachses, *Biochem. Z.*, 169 : 13–66 (1926).

[8] Nelson, E. K., Acetyl Groups in Pectin, *J. Am. Chem. Soc.*, 48 : 2945–2946 (1926).

[9] Carré, M. H., and Haynes, D., The Estimation of Pectin as Calcium Pectate and the Application of This Method to the Determination of the Soluble Pectin in Apples, *Biochem. J.*, 16 : 60–69 (1922).

[10] Carré, M. H., Chemical Studies in the Physiology of Apples. IV. Investigations on the Pectic Constituents of Apples, *Ann. Bot.*, 39 : 811–839 (1925).

that both of these substances are combined with cellulose; that cellulose is actually esterified with some of the galacturonic acid groups; that in the middle-lamella pectin there is more cellulose and less methoxy groups, and in the cell-wall thickenings there is less cellulose and more methoxy groups in combination with the pectin radical. Thus, Schweitzer's reagent will completely dissolve away all cellulose substances and leave the middle lamella as a fine network. This network then readily dissolves in weak acid followed by weak alkali. Sucharipa[11] has prepared a material by Schweitzer's reagent which he believes to be protopectin.

Protopectin can be hydrolyzed free from the cellulose of the wall and converted into soluble "pectin" by several means:—(1) by long continued boiling with water, especially under pressure; (2) by treatment with 0.5 per cent ammonium oxalate at 70–90°; (3) by action of the enzyme, protopectinase. The second method is the one most frequently employed. The transformation also occurs during the ripening of fruits. However it is brought about, it results in the separation of the cells from each other and is usually spoken of as "maceration."

The resultant pectin is the best known of all the pectic substances, and is the pectin of commerce. It is a material of high molecular weight; it disperses in water to a viscous colloidal sol; it is readily precipitated from this sol by alcohol, which acts as a dehydrating agent, by lead, iron, and other heavy metal salts, but not by salts of calcium. Its most outstanding property is its ability to form sugar-acid-pectin gels, and fruit jellies of this nature have been long known.

The purest preparations of pectin contain from 10 to 12 per cent of methyl alcohol combined as methoxy groups or as methyl ester groups. Demethoxylation occurs slowly when the pectin is boiled with water or dilute acid; but it occurs within a few minutes at room temperatures when pectin is treated with dilute alkali. In fact this saponification constitutes a simple method for determining the methoxy content.

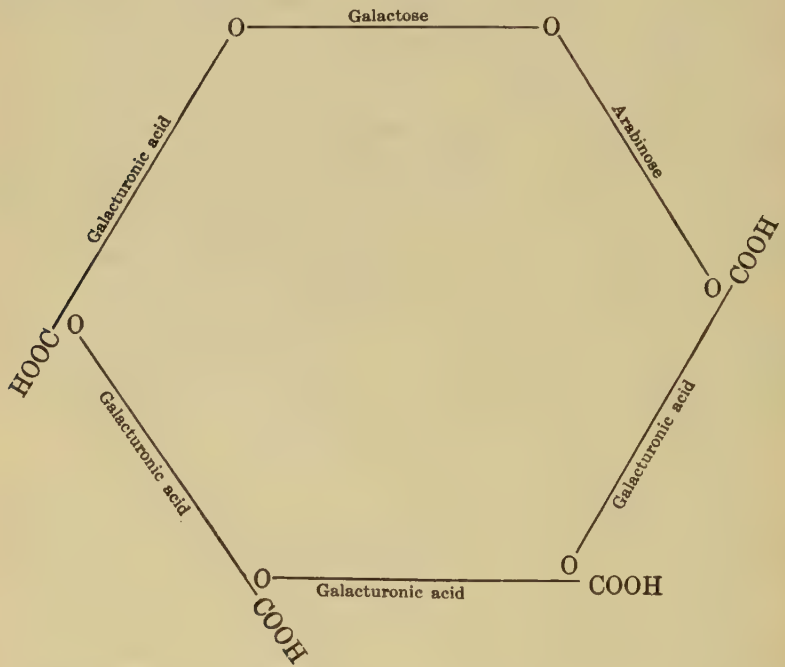
This demethoxylated pectin is pectic acid.[12] Probably the purest preparations of pectic substances so far described have been of this pectic acid, because of the facts that it is a simpler substance and that its solubilities enable it to be purified more readily. The usual procedure is to prepare pectin by repeated precipitations with alcohol; saponify the pectin with dilute sodium hydroxide, producing soluble sodium pectate; acidify with hydrochloric acid, producing the gelatinous, insoluble pectic

[11] Sucharipa, R., Protopectin and Some Other Constituents of Lemon Peel, *J. Am. Chem. Soc.*, 46 : 145–156 (1924).

[12] The term, "pectinic acids," has been revived by Carré to apply to compounds of intermediate methoxy content, *i.e.*, between pectin and pectic acid.

acid; and dry this with alcohol and ether. Or the saponification may be brought about by limewater, producing the gelatinous, insoluble calcium pectate. This gel may be freed from calcium by treating with ammonium oxalate, inasmuch as the ammonium pectate is soluble.

In 1916, Schryver and Haynes[13] prepared pectic acid from four different sources, and found that all four agreed with the empirical formula $(C_{17}H_{24}O_{16})_x$. Later Carré and Haynes prepared a calcium pectate that agreed well with this formula. Then, Nanji, Paton, and Ling isolated what they consider to be the basic unit of the pectins, a substance with the empirical formula $(C_{35}H_{50}O_{33})_x$. This again agrees well with Schryver and Haynes' formula. Furthermore, from analysis of the constituent sugars and galacturonic acid they proposed the following structure, consisting of four molecules of galacturonic acid, one of galactose, and one of arabinose, arranged in a ring, with the carboxyls of the acid groups free and available for methyl ester formation. The specific order of the units in the ring was not suggested.



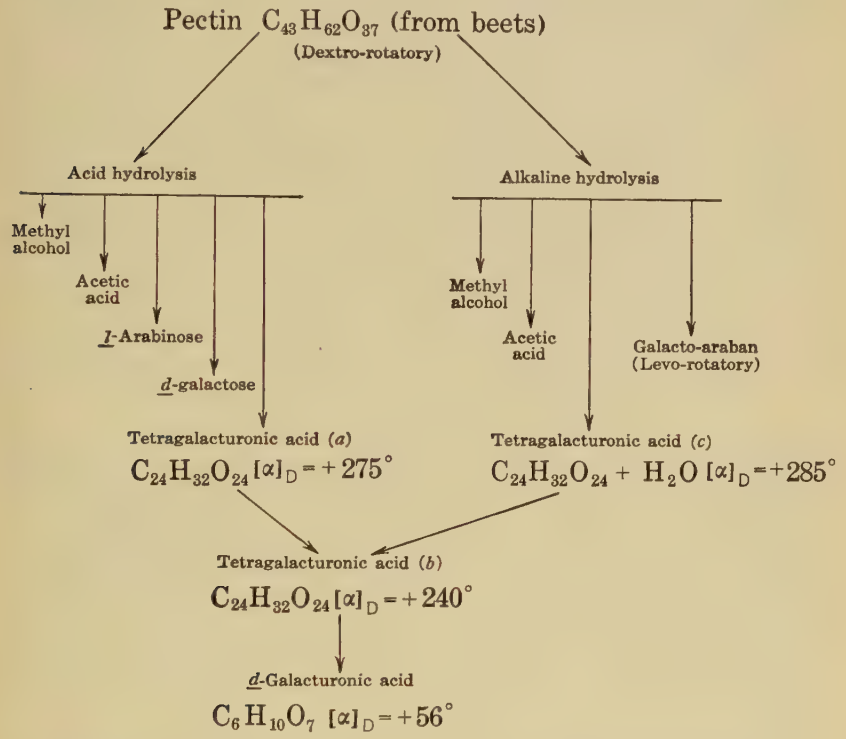
Ehrlich,[14] after careful work on beet and flax pectin covering several

[13] Schryver, S. B., and Haynes, D., The Pectic Substances of Plants, *Biochem. J.*, 10 : 539–547 (1916).

[14] Ehrlich, F., Neue Untersuchungen über Pektinstoffe, *Z. angew. Chem.*, 40 : 1305–1313 (1927).

years, found the same constituents, and in the same proportions, as those in the above formula. However, he isolated a tetragalacturonic acid of closed-ring structure, which led him to the conclusion that the sugar acids and sugars are not in the same ring, as in the above formula.

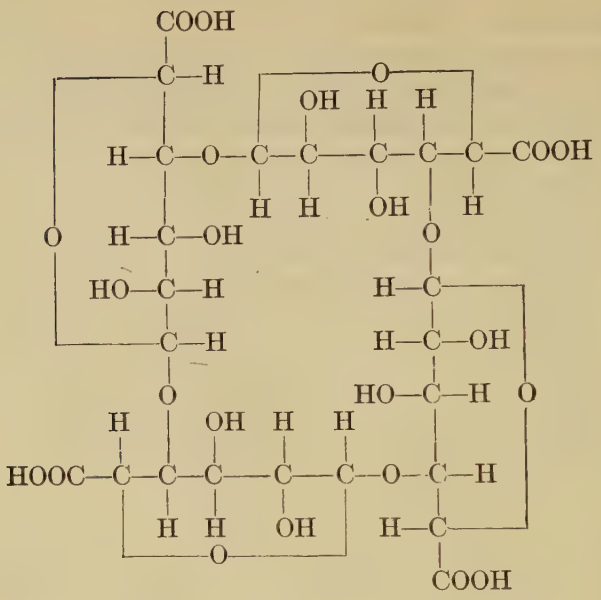
Ehrlich diagrams the decomposition products of beet pectin as follows:



The galactose-arabinose disaccharide was isolated, indicating that both sugars are not connected directly to the ring.

On this basis the nucleus of pectic acid appears to be a new type of carbohydrate derivative, *i.e.*, a tetragalacturonic acid in which four molecules of galacturonic acid are condensed with the elimination of four molecules of water. Each of the four aldehyde groups is linked to a hydroxyl group in another molecule, and all four of the carboxyl groups remain free to be esterified with methyl alcohol or to form metallic salts. The galacto-arabinose disaccharide appears to be linked to the tetragalacturonic acid through one of the hydroxyls of the latter.

Such a nucleus would have approximately the following formula:



Ehrlich's tetragalacturonic acid, $C_{24}H_{32}O_{24}$, (position of oxygen linkages not certain)

Ehrlich believes that the pectin from sugar beets is a triacetyl-arabino-galacto-dimethoxy-tetragalacturonic acid ($C_{43}H_{62}O_{37}$), and yields as hydrolytic products four molecules of galacturonic acid, two of methanol, three of acetic acid, and one each of arabinose and galactose. The pectin from flax straw differs from this in having but two molecules of acetic acid, and in having in addition one molecule of xylose. This pectin he designates as diacetyl-arabino-xylo-galacto-dimethoxy-tetra-galacturonic acid ($C_{46}H_{68}O_{40}$). The positions of attachment of these various molecules have not been determined.

The important point of comparison in the above suggested formulae is not in the disagreement of the structure, but in the really close agreement in the kind and quantity of constituent groups. This augurs well for the future elucidation of the structure of the pectic substances.

Commercial Pectin.—The introduction of commercial pectin has made possible the production, either in the home or in the factory, of jellies from fruits which contain too little pectin of their own; it has made possible the standardization of jelly making; and it eliminates the long cooking of delicately flavored fruits.

Most commercial pectin is prepared either from apple pomace or cull lemons. Table LIX shows the quantities of pectin in these and in other

TABLE LIX

Percentage of Pectin in Raw Materials

	Fresh Material	Dry Matter Basis
Apple pomace	1.5–2.5	15.0–18.0
Lemon pulp	2.5–4.0	30.0–35.0
Orange pulp	3.5–5.5	30.0–40.0
Beet pulp	1.0	25.0–30.0
Carrots	0.62	7.14

materials suggested as sources of pectin.[15] The pulp, either apple or lemon, is given a preliminary washing, and is then cooked with water, usually containing sulfurous acid, to bring the pectin into solution. The filtered extract may be evaporated to a syrupy solution and sold as liquid pectin, or the pectin may be precipitated by aluminum sulfate and ammonia. This precipitate is dried, and the aluminum removed by washing with acidified alcohol. The final purified product is sold as powdered pectin. A more recent development is the precipitation of the pectin by alcohol instead of by alum.

Pectin is usually sold according to its "jelly grade," which is the number of pounds of sucrose that one pound of pectin can "carry" in a jelly of standard acidity and water content. This jelly grade varies from 100 to 230.

A gel made with pectin requires three ingredients in proper concentration,—pectin, sugar (usually sucrose), and acid. The sugar must be present to the extent of 65 to 70 per cent, and the acidity must be equivalent to a *p*H of 3.2 to 3.5.[16-21] This acidity is attained by the use of

[15] Wilson, C. P., The Manufacture of Pectin, *Ind. Eng. Chem.*, 17 : 1065–1067 (1925).

[16] Tarr, L. W., Fruit Jellies. I. The Role of Acids, Bull. No. 134, Delaware Agr. Exp. Sta. (1923).

[17] Tarr, L. W., and Baker, G. L., Fruit Jellies. II. The Role of Sugar, Bull. No. 136, Delaware Agr. Exp. Sta. (1924).

[18] Tarr, L. W., Fruit Jellies. III. Jelly Strength Measurements, Bull. No. 142, Delaware Agr. Exp. Sta. (1926).

[19] Myers P. B., and Baker, G. L., Fruit Jellies. IV. The Role of Salts, Bull. No. 144, Delaware Agr. Exp. Sta. (1926).

[20] Myers, P. B., and Baker, G. L., Fruit Jellies. V. The Role of Pectin. I. The Viscosity and Jellying Properties of Pectin Solutions, Bull. No. 149, Delaware Agr. Exp. Sta. (1927).

[21] Myers, P. B., and Baker, G. L., Fruit Jellies. VI. The Role of Pectin. II. The Extraction of Pectin from Pectic Materials. Bull. No. 160, Delaware Agr. Exp. Sta. (1929).

approximately 0.052 per cent tartaric, and 0.066 per cent citric, acid in the final gel. The quantity of pectin required depends, of course, upon its quality, as noted above. It may be as low as 0.3 and as high as 0.7 per cent.

Quantitative Estimation of Pectic Substances.—The commonest and simplest method of determining pectin is to precipitate the boiled and filtered solution with alcohol. The precipitate is then washed with alcohol, dried, and weighed. This eliminates most of the proteins, but not the polysaccharides, such as starch and gums.

The method devised by Carré and coworkers[22] for the estimation of pectin is the most definite of any yet proposed. The pectin is saponified with 0.1 N sodium hydroxide, acidified with acetic acid, and the pectic acid precipitated as the calcium salt by means of calcium chloride. The calcium pectate is very insoluble, and can be washed very free from impurities. As prepared from apple pectin it has a composition corresponding to the formula, $C_{17}H_{22}O_{16}Ca$.

Miss Carré has extended this determination to apply to all pectic substances, and has used it in extensive investigations on apples.[23] The finely pulped tissue is first extracted with cold water to remove the pectin. The residue is boiled with 0.0133 M hydrochloric acid for three to five periods of three hours each. This dissolves the pectose, which occurs as thickenings on the cell walls. The dissolved material is then saponified and converted into calcium pectate as outlined above. The residue, containing now only the pectic substance of the middle lamella, is boiled with 0.0133 M sodium hydroxide for several periods of one-half hour each. This dissolves the middle lamella and converts it into pectic acid, which is determined as before as calcium pectate.

Another method that has been suggested for determining the pectic substances as a whole consists in measuring the carbon dioxide produced by the decarboxylation of the uronic acids when the material is boiled with 12 per cent hydrochloric acid.[24] Although this is apparently a quantitative measure of the uronic acid content, these acids are found in other substances which may occur associated with the pectins.

[22] Emmett, A. M., and Carré, M. H., A Modification of the Calcium Pectate Method for the Estimation of Pectin, *Biochem. J.*, 20 : 6–12 (1926). [Cf. *Biochem. J.*, 16 : 60–69 (1922).]

[23] Carré, M. H., An Investigation of the Changes Which Occur in the Pectic Constituents of Stored Fruits, *Biochem. J.*, 16 : 704–712 (1922). [Cf. *Ann. Bot.*, 39 : 811–839 (1925).]

[24] Dore, W. H., The Composition of Pectin: A Preliminary Report of the Determination of Galacturonic Acid in Pectin, *J. Am. Chem. Soc.*, 48 : 232–236 (1926). (Cf. Nanji, Paton, and Ling, *loc. cit.*)

Action of Enzymes on Pectins.—There are generally recognized three pectic enzymes,[25] (1) protopectinase, (2) pectase, and (3) pectinase.

Protopectinase hydrolyzes the middle lamella substance away from the cellulose walls, thus macerating the tissue. Presumably the product formed is pectin, although this has never been prepared from the middle lamella by enzymic process. The enzyme is demonstrated only by its macerating action on plant tissue, usually a storage tissue like tubers, fleshy roots, and fruit.

Pectase is an esterase, and hydrolyzes the methoxy groups from pectin, producing pectic acid. It is demonstrated by allowing it to act on pectin in the presence of calcium, when a gel of calcium pectate is produced.

Pectinase hydrolyzes pectin (and possibly pectic acid) to its simple components, sugars and galacturonic acid, and is demonstrated by the formation of these reducing substances.

It is not known what enzyme hydrolyzes acetic acid away from pectin.

Protopectinase has so far been found largely in certain parasitic fungi. Its function is probably to enable the fungus to penetrate the tissue by dissolving the middle lamella.

The function of pectase is more obscure. It is found most abundantly in leaves, and to a lesser extent in certain fungi. Bertrand and Mallèvre[26-28] found it in a large number of plants, mostly in tissues where growth was vigorous.

Pectinase is obviously a digestive enzyme secreted by fungi for making available the constituents of pectin.

The relations among the pectic substances, their interconversion by means of chemicals, and the action of their enzymes are shown in the diagram on the following page.

It should be pointed out that there are two steps in this scheme which have not as yet been demonstrated. One is the enzymic hydrolysis of pectose. We do not know whether protopectinase will accomplish this, or whether pectose has its own enzyme. This is on the assumption, of course, that Carré's pectose is different from the middle lamella protopectin. The other gap in our knowledge is the action of pectinase.

[25] Davison, F. R., and Willaman, J. J., Biochemistry of Plant Diseases. IX. Pectic Enzymes, *Bot. Gaz.*, 83 : 329–361 (1927).

[26] Bertrand, G., and Mallèvre, A., Sur la pectose et sur la fermentation pectique, *Compt. rend.*, 119 : 1012–1014 (1894).

[27] Bertrand, G., and Mallèvre, A., Nouvelles recherches sur la pectose et sur la fermentation pectique, *Compt. rend.*, 120 : 110–112 (1895).

[28] Bertrand, G., and Mallèvre, A., Sur la diffusion de la pectose dans le règne végétal et sur la préparation de cette diastase, *Compt. rend.*, 121 : 726–728 (1895).

Investigators have always used pectin for its substrate. The fate of the methanol in this case is unknown. Whether pectinase can act on pectic acid, or on one of its soluble salts is not known.

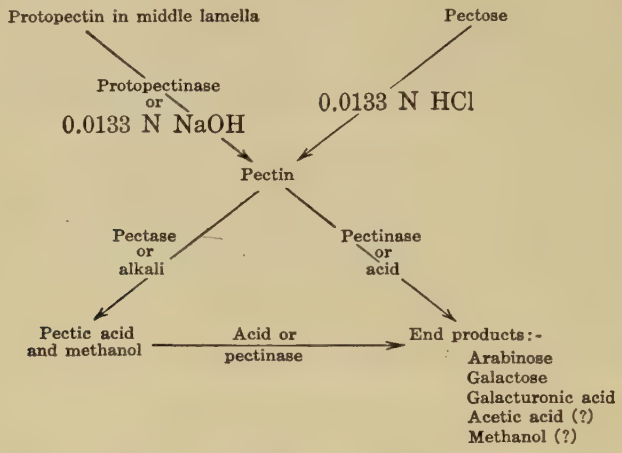


Table LX presents a brief summary of the occurrence of the pectic enzymes.

TABLE LX

Occurrence of Pectic Enzymes. Partial List

(+ = present; − = absent; --- = not tested)

Source	Proto-pectinase	Pectase	Pectinase
Rhizopus tritici	+	−	+
Rhizopus oryzae	+	+	+
Rhizopus nigricans	+	+	+
Aspergillus niger	−	−	+
Clostridium sp.	+	+	+
Sclerotinia libertiana	+	+	+
Sclerotinia cinerea	−	+	+
Trifolium pratense (leaves)	−	+	−
Nicotiana tabacum (leaves)	---	+	---
Takadiastase	+	−	+
Zea mays (pollen)	−	+	−
Barley malt	−	+	+
Emulsin	+	−	+
Mespilus germanica (fruit)	+	+	−
Daucus carota (root)	−	+	−
Syringa vulgaris (leaves)	---	+	---
Solanum tuberosum (leaves)	---	+	---

Biological Significance of Pectin.—The pectic substance of the middle lamella apparently functions as a cementing material between cells. During the ripening of fruit this lamella is dissolved, the cells separate from each other, and the tissue disintegrates. The retting of

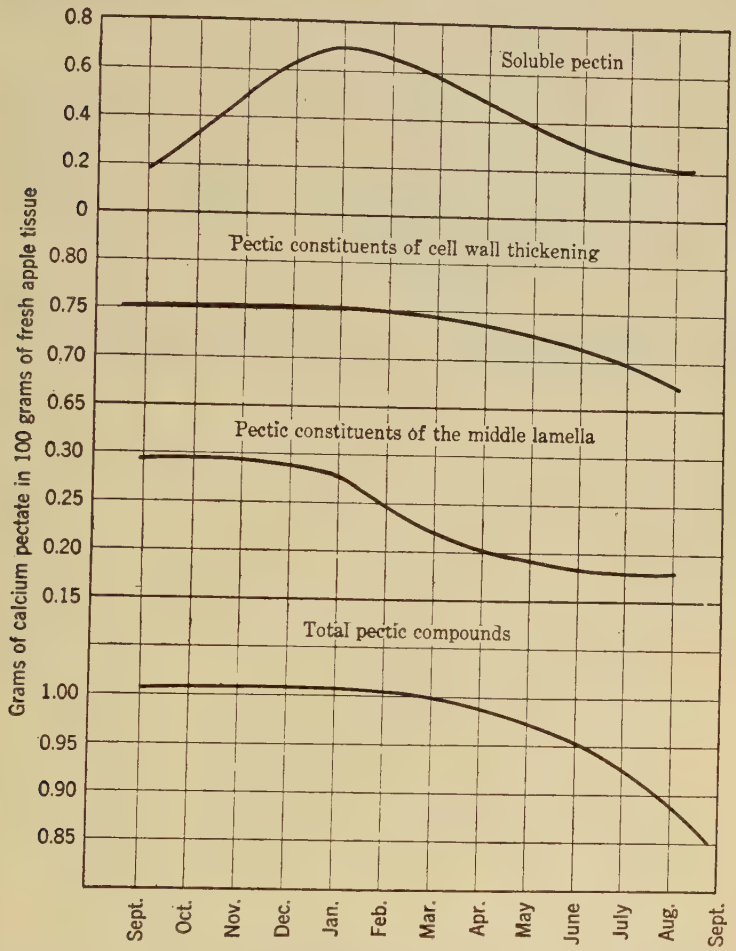


Fig. 125.—Showing the pectic changes which take place in apples from the time of picking to the last stages of senescence. (Data of Carré and Horne.)

flax has been shown to be a bacterial dissolution of the pectic material between fibers.

Carré and Horne[29] have followed in great detail the changes in the

[29] Carré, M. H., and Horne, A. S., An Investigation of the Behaviour of Pectic Materials in Apples and Other Plant Tissues, *Ann. Bot.*, 41 : 193–237 (1927).

pectic substances of apples during the after-ripening period in cold storage. They used the chemical methods outlined above for distinguishing pectin, pectose, and the middle lamella substance, and also microchemical methods based on the specific staining of pectic substances by ruthenium red [$Ru_2(OH)_2Cl_4 \cdot 7(NH_3) \cdot 3H_2O$]. The changes which they found are shown in Fig. 125.

Appleman and Conrad[30,31] found that during the ripening of peaches the total pectic substances remained constant, but that there was a decided decrease in protopectin and a corresponding increase in pectin. They found that in tomatoes this change is very rapid, and that the disintegration of the fruit during the canning process is the greatest when the ratio of pectin to protopectin is greatest.

[30] Appleman, C. O., and Conrad, C. M., Pectic Constituents of Peaches and Their Relation to Softening of the Fruit, Bull. No. 283, Maryland Agr. Exp. Sta. (1926).

[31] Appleman, C. O., and Conrad, C. M., The Pectic Constituents of Tomatoes and Their Relation to the Canned Product, Bull. No. 291, Maryland Agr. Exp. Sta., (1927).

CHAPTER XXVIII

THE TANNINS

TANNIN is a generic name for a group of substances widely distributed in the higher plants and showing certain characteristic physical and chemical properties. Tannins may be found in leaves, tea (15 per cent), sumac, *Rhus coriaria* (13–15 per cent), rhododendron, etc. They occur also in wood and stems, and especially in the bark of the oaks, hemlock, etc.; in fruits, especially unripe fruits, *e.g.*, persimmons, plums, hulls of walnuts and of butter nuts and hickory nuts; in seeds (more rarely), but especially in the seed coats; in algae, fungi, and pathological plant growths. Plant galls may contain as much as 75 per cent tannin and rarely contain less than 25 per cent.

Only one instance is recorded of a tannin from an animal source. Three per cent of a substance was extracted from corn weevils which, on hydrolysis with dilute acids, yielded dextrose, gallic acid, and a red "phlobaphene." The existence of a true animal tannin may well be doubted, for this product may have been derived from undigested food remains.

Tannins may be characterized by the following properties:

1. They are amorphous (non-crystalline).
2. They have an astringent taste.
3. They give colors (inks) with ferric salts.
4. They are precipitated from solution by $K_2Cr_2O_7$ and by alkaloids.
5. They precipitate gelatin from solution, which property enables them to convert hide into leather.
6. Their sols develop a deep red color on the addition of potassium ferricyanide.
7. They are readily soluble in hot water to form solutions which are in reality colloidal sols.
8. They are hydrolyzed by acids into a variety of products, one being nearly always a sugar (usually *d*-glucose) and the other a hydroxy derivative of the aromatic series (usually an acid). A few yield phloroglucinol or some similar phenol instead of the sugar. While tannins usually contain sugar, they do not show the characteristic properties of the true glucosides and should be considered as a separate class.

9. They may act as a chromogen for oxidases, *e.g.*, the green walnut hulls when broken open darken at once, due to oxidation of tannin by oxidases or even by exposure to air.

Haas and Hill[1] state, " While the composition of the various classes of tannins of course varies considerably, they are probably all more or less complex derivatives of gallic or ellagic acids, or their methylated derivatives, or are condensation products of these or similar acids, with various phenolic substances."

The classification and properties, in the light of the above definition, can be better understood if we consider first certain of the relatively simple phenols from which the more complex tannins are built up.

Procter[2] uses a classification into (A) pyrogallol tannins and (B) pyrocatechol tannins.

Reagent	Pyrogallol Tannins, Reaction	Pyrocatechol Tannins, Reaction
Ferric salts	Dark blue	Green-black
Bromine water	No precipitate	Yellow or brown precipitate
Finish on leather	Produces a " bloom," consisting of ellagic acid, on leather	No bloom
Concentrated sulfuric acid	.....	Dark red ring at juncture of liquids

Certain of the pyrocatechol tannins contain phloroglucinol as a component, notably the " gambier " tannins.

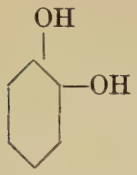
When tannins are heated with dilute acids, insoluble amorphous anhydrides or " *phlobaphenes* " (apparently anhydrides of the tannin) are produced besides the sugars, gallic acid, and ellagic acid. These phlobaphenes are produced by any process tending to cause the tannin to lose water. They are red or brown colored substances, practically insoluble in water, chemically relatively inert, and occur in nature only associated with the tannins.

The " tannin " of commerce is gallotannic acid or digallic acid, an amorphous powder, soluble in water, acid to litmus, soluble in alcohol and glycerol, but only slightly soluble in other organic solvents, such as ether, benzene, carbon bisulfide, etc. It possesses a very astringent taste.

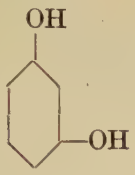
[1] Haas, P., and Hill, T. G., An Introduction to the Chemistry of Plant Products Vol. I, Third Edition, Longmans, Green and Company, London (1921).

[2] Procter, H. R., The Principles of Leather Manufacture, Spon and Chamberlain, New York (1903).

1. Dihydroxyphenols



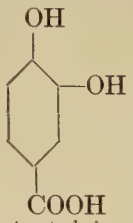
Pyrocatechol



Resorcinol

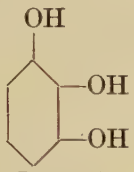
Hydroquinone

2. Dihydroxy acid

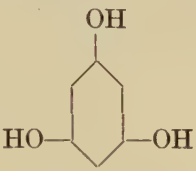


Protocatechuic acid

3. Trihydroxyphenols

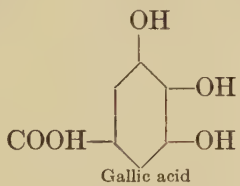


Pyrogallol



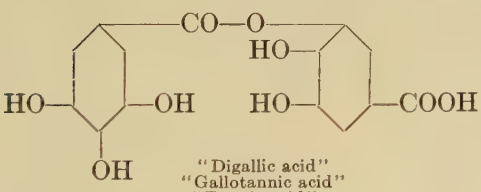
Phloroglucinol

4. Trihydroxy acid

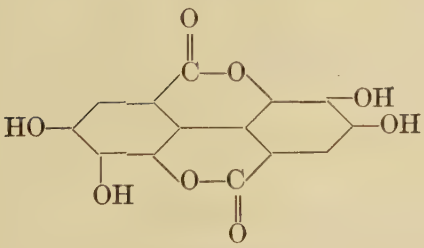


Gallic acid

$POCl_3$ or other dehydrating agents



"Digallic acid"
"Gallotannic acid"
"Tannic acid"



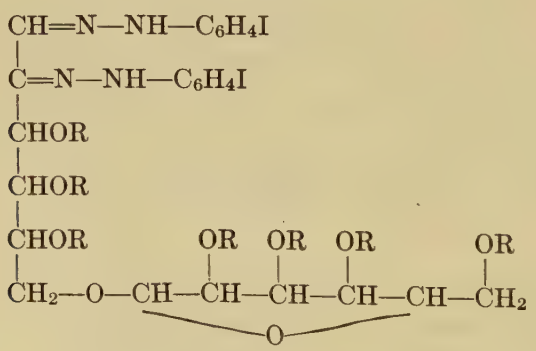
Ellagic acid
(The dilactone of the diphenyl derivative corresponding to gallic acid)

Emil Fischer solved the problem as to whether or not sugar was an integral part of the tannin molecule by purifying the tannin from Chinese nut galls and then hydrolyzing it in acid solution. He obtained one molecule of glucose for each ten molecules of gallic acid, and concluded that the Chinese nut gall tannin corresponded to pentadigallyl glucose, *i.e.*, one molecule of digallic acid replacing each acetyl group in pentaacetyl glucose. The theory indicates 10.6 per cent of glucose in such a molecule, and Fischer and Freudenberg [3] found about 8 per cent in a tannin which had been carefully purified.

The same workers synthesized a pentagallyl glucose, and Fischer believes that such compounds may well exist in stereoisomers corresponding somewhat to the α- and β-glucosides. Two pentabenzoyl glucoses are known.

Fischer's synthesis must be understood as applying definitely to only one kind of tannin, *i.e.*, that similar to the Chinese nut gall tannin. It apparently does not belong to the same group as the hemlock tannins which do not appear to contain sugars. The probability, however, is that Fischer's general idea will hold for most of the tannins.

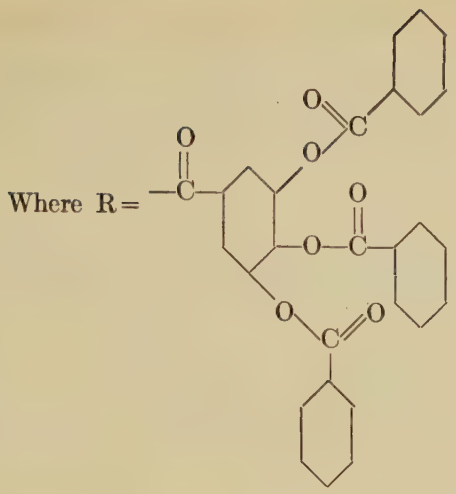
In the course of their work, Fischer and Freudenberg [4] prepared hepta (tribenzoylgalloyl) *p*-iodiophenyl maltosazone, $C_{220}H_{142}O_{58}N_4I_2$, which has a molecular weight of 4021. This compound has the highest molecular weight of any compound which has so far been synthesized. We have already noted Fischer's octadecapeptide, $C_{52}H_{88}O_{19}N_{18}$, which had a molecular weight of only 1408.



Hepta (tribenzoylgalloyl) *p*-iodiophenyl maltosazone

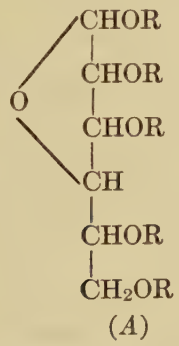
[3] Fischer, E., and Freudenberg, K., Über das Tannin und die Synthese ähnlicher Stoffe I., *Ber.*, 45 : 915–935 (1912).

[4] Fischer, E., and Freudenberg, K., Über das Tannin und die Synthese ähnlicher Stoffe III., Hochmolekulare Verbindungen, *Ber.*, 46 : 1116–1138 (1913).



The iodine was deliberately introduced into the molecule in order to provide for an accurate analysis, since in such a large molecule the analysis for carbon and hydrogen alone would not be sufficiently accurate to determine structural relationships.

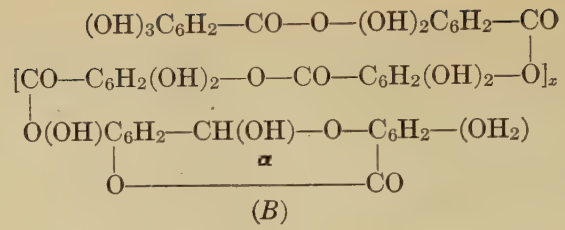
Nierenstein, Spiers, and Hadley[5] have suggested another formula based on the evidence that all of the glucose in gallotannin can be fermented off without disrupting the tannin molecule. The resulting tannin does not yield glucose on hydrolysis, but otherwise appears to give all of the properties of gallotannin, *i.e.*, it is precipitated by gelatin and quinine and is adsorbed by casein and hide powder. They conclude, therefore, that Fischer's formula (*A*) cannot be correct, and they propose instead the formula (*B*).



(*A*)

Where R = *m*-digallic acid.

[5] Nierenstein, M., Spiers, C. W., and Hadley, A. C., Gallotannin. XIV. The Action of Yeast on Gallotannin, *J. Am. Chem. Soc.*, 47 : 1726–1728 (1925).



(*B*)

where a glucose radical can be attached to the (OH_α) in a glucoside type of union, the non-sugar residue being a polydigalloyl-leucodigallic acid anhydride.

The process of tanning may be regarded as the conversion of a relatively hydrophilic colloid gel into a relatively non-hydrophilic gel which we call "leather." Undoubtedly both chemical and physical processes enter into the reactions. Wilson,[6] and Thomas,[7-13] have during the past few years attempted by the use of physico-chemical technic, to analyze at least a part of the variables involved in the conversion of collagen to leather.

The technic employed for the evaluation of a tannin extract gives a definition of tannin from a practical viewpoint, *i.e.*, that portion of the water-soluble matter of certain vegetable materials which will precipitate gelatin from solution and which will combine with hide fibers to form compounds which are resistant to washing. The remaining portion of water-soluble materials is called non-tannins.

The tannin-containing "solution" is shaken with purified hide powder until all of the tannin has been removed from solution. This

[6] Wilson, J. A., The Chemistry of Leather Manufacture, Am. Chem. Soc. Monograph No. 12, Chemical Catalog Company, Inc., New York (1923); cf. also Second Edition, Vol. I, *ibid.* (1928).

[7] Thomas, A. W., and Foster, S. B., The Electrical Charge of Vegetable Tannin Particles, *Ind. Eng. Chem.*, 15 : 707–708 (1923).

[8] Thomas, A.W., and Frieden, A., The Gelatin-Tannin Reaction, *Ind. Eng. Chem.*, 15 : 839–841 (1923).

[9] Thomas, A. W., and Kelly, M. W., Concentration Factor in the Combination of Tannin with Hide Substance, *Ind. Eng. Chem.*, 15 : 928 (1923).

[10] Thomas, A. W., and Kelly, M. W., The Influence of Hydrogen-Ion Concentration in the Fixation of Vegetable Tannins by Hide Substance, *Ind. Eng. Chem.*, 15 : 1148–1153 (1923).

[11] Thomas, A. W., and Kelly, M. W., The Influence of Neutral Salts upon the Fixation of Tannin by Hide Substance, *Ind. Eng. Chem.*, 15 : 1262–1263 (1923).

[12] Thomas, A. W., and Kelly, M. W., Tannic Acid Tannage, *Ind. Eng. Chem.*, 16 : 800–802 (1924).

[13] Thomas, A. W., and Kelly, M. W., Vegetable Tanning, *Ind. Eng. Chem.*, 17 : 41–43 (1925).

point is ascertained by testing filtered portions with gelatin to see if a precipitate still forms (cf. Wilson and Kern[14]).

Several substitutes for tannin in leather manufacture have been suggested. These have been called synthetic tannins, *i.e.*, substances which tan hides but they may or may not have a chemical structure analogous to natural tannins. Stiasny[15-18] has prepared such a "synthetic tannin" from a phenol treated with sulfuric acid and formaldehyde in the proportion of one molecule of formaldehyde to two molecules of phenol. The method of preparation suggests "bakelite" or "redmanol," but this substance is water-soluble. It is claimed to make a good leather and to require a shorter tanning period than is necessary when the natural tannins are used.

Meunier and Seyewetz (1908) used quinone. They found that when gelatin was treated with phenols under conditions suitable for oxidation, the resulting precipitate became insoluble in boiling water. They state that, "quinonated gelatin constitutes the most stable form of insoluble gelatin thus far known," since it resists not only boiling water but also dilute acids and alkalies. Only two hours at 15° C. are required to render the gelatin completely insoluble. Thomas and Kelly[19] note that the optimum conditions for quinone tanning require an alkaline solution, approximately *p*H 8–10.

Physiological Significance of the Tannins.—Plant physiologists differ widely in their views as to the function of tannins in plants. Pfeffer believed that they served some useful purpose and were not merely by-products. He did not consider that they were necessary, however, to furnish benzene rings from which other compounds, such as proteins, etc., might be synthesized. Neither did he consider them as essential for cell activity. Moore suggests that the plant builds up tannins as a means of neutralizing acids by esterification, and Fischer's syntheses lend some support to this view. Kraus found tannins to be a direct synthetic product formed only in sunlight and carbon dioxide supply, and translocated to stems, bark, and roots. Sachs con-

[14] Wilson, J. A., and Kern, E. J., The True Tanning Value of Vegetable Tanning Materials, *J. Ind. Eng. Chem.*, 12 : 465–469 (1920).

[15] Stiasny, E., A Synthetic Tannin, *Leather World*, 12 : 227 (1913).

[16] Stiasny, E., Syntans, New Artificial Tanning Materials, *J. Soc. Chem. Ind.*, 32 : 775–777 (1913).

[17] Diedorf, H., Neradol D and Its Practical Applications, *J. Am. Leather Chem. Assoc.*, 8 : 394–404 (1913).

[18] Nihoul, E., The Synthetic Tannins and Their Use in Tanning, *Sci. Am. Monthly*, 1 : 326–330 (1920).

[19] Thomas, A. W., and Kelly, M. W., Quinone Tannage, *Ind. Eng. Chem.*, 16 : 925–926 (1924).

cluded that tannins occur (are produced) in the regions of most intense metabolic activity, such as in active leaves, in rapid tissue formation, such as galls, and in other pathological growths, and as a consequence of some particular stimulation. Drabble and Nierenstein conclude that cork formation is associated with tannins and that in the plant cells the complex phenols are acted upon by acids and formaldehyde, so that they are precipitated in the " cork " cell.

Other workers believe that cell wall formation or spore formation is associated, to a greater or less extent, with tannins. It has even been suggested that tannins are a special protection of the plant against animals! One of the more recent views is that they act as an antiseptic in case of wound formation, especially as a protection against fungi (cf. Cook and Taubenhaus[20]). These workers point out that fungi are quite sensitive to tannins and that apparently the parasitic fungi are more sensitive than the saprophytic forms. It is true that tannin collects in wounded tissue in abnormally large amounts.

In green fruits tannin is more or less abundant but apparently disappears as the fruit ripens. It may not actually disappear but instead be "locked up " in giant cells in a more or less insoluble form. This seems to be the mechanism in the persimmon (cf. Gore[21, 22]).

The Japanese " process " the persimmons by placing the unripe fruit in casks from which their rice wine (sake) has just been drawn. In five to fifteen days the casks are opened and the astringent taste will have wholly disappeared. Gore used other anaesthetics, such as ether, chloroform, etc., to produce the same result. It was found that the tannin was localized in giant cells, some large enough to be seen with the naked eye. In the partly ripe persimmon, these cells do not break as soon as taken into the mouth but swell and eventually burst, emptying their thick tannin-bearing contents on the tongue. In the *processed* or *ripe* persimmons the contents of these cells have undergone a change, becoming more refractive and hard and losing their imbibitional power, so that they swell but slightly and do not burst. The loss of astringency presumably is due to the hardening of the contents of these giant cells.

[20] Cook, M. T., and Taubenhaus, J. J., The Relation of Parasitic Fungi to the Contents of the Cells of the Host Plants. I. The Toxicity of Tannin, Bull. No. 91; II. The Toxicity of Vegetable Acids and the Oxidizing Enzyme, Bull. No. 97, Delaware Agr. Exp. Sta. (1911–1912).

[21] Gore, H. C., Experiments on the Processing of Persimmons to Render Them Nonastringent, Bull. No. 141, Bur. of Chem., U. S. Dept. of Agr. (1911).

[22] Gore, H. C., Large Scale Experiments on the Processing of Japanese Persimmons, Bull, No. 155, Bur. of Chem., U. S. Dept. of Agr. (1912).

CHAPTER XXIX

CHLOROPHYLL AND ASSOCIATED PIGMENTS

Chlorophyll is the green coloring material of plants and is almost always accompanied by two yellow pigments, carotin ($C_{40}H_{56}$) and xanthophyll ($C_{40}H_{56}O_2$).

Chemists from the time of Berzelius (1839) have struggled with the chemistry of chlorophyll, and its chemical nature has finally been fairly well solved by Willstätter and his coworkers (cf. Willstätter and Stoll,[1] West,[2] Willstätter,[3] and Stewart[4]).

Chlorophyll probably exists in the colloidal state in plants, or at least adsorbed upon colloids. It can be extracted with certain organic solvents. The earlier workers thought that various plants were characterized by different varieties of chlorophyll. Willstätter has shown, however, that there is only one variety. This exists, at least as isolated in the laboratory, in two forms which have been designated as chlorophyll *a* and chlorophyll *b*.

Chlorophyll, possessing the same properties, may be prepared from either fresh or dried leaves. One kilo of fresh leaves gives a yield of from 0.9 to 2.1 grams, while dried leaves yield 5 to 10 grams. The most suitable solvent for extraction is acetone (80 per cent) for dried leaves, and pure acetone for fresh leaves, sufficient acetone being added so that, allowing for the moisture in the fresh leaves, the resulting solution is 80 per cent acetone. Willstätter states that chlorophyll can be now isolated as readily as can any alkaloid or any sugar, and that within a few hours a kilo of dried leaves should yield about 6.5 grams of practically pure chlorophyll (cf. Schertz[5-7]).

[1] Willstätter, R., and Stoll, A., Untersuchungen über Chlorophyll, Julius Springer, Berlin (1913).

[2] West, C. J., A Review of Willstätter's Researches on Chlorophyll, *Biochem. Bull.*, 3 : 229–258 (1914).

[3] Willstätter, R., Chlorophyll, *J. Am. Chem. Soc.*, 37 : 323–345 (1915).

[4] Stewart, A. W., Recent Advances in Organic Chemistry, Third Edition, Longmans, Green and Company, London (1918).

[5] Schertz, F. M., The Extraction and Separation of Chlorophyll ($\alpha + \beta$), Carotin and Xanthophyll in Fresh Green Leaves, Preliminary to Their Quantitative Determination, *Plant Physiol.*, 3 : 211–216 (1928).

Chloropyll is a bluish-black substance with a strong metallic luster, powdering to a greenish- or bluish-black powder. It has no definite melting point, ranging from 93° to 106° C. for various samples, and is soluble in absolute alcohol to a blue-green solution. It shows neither acidic nor basic properties. Acids change its color to olive brown and split off magnesium which is associated with the molecule.

Pure chlorophyll shows the following characteristics:

1. The ash content is 4.5 per cent, the ash being pure magnesium oxide.

2. One-third of the molecule is accounted for in the form of a monoatomic alcohol, phytol, $C_{20}H_{39}OH$.

3. The associated yellow pigments are not a part of the chlorophyll molecule.

4. When chlorophyll is saponified with potassium hydroxide in methyl alcohol, the color changes to a pure brown; impure mixtures give a dirty brown.

5. Chlorophyll is decomposed by boiling alcoholic potassium hydroxide, and there is formed a normal mixture of " phytochlorine *e* " and " phytorhodin *g*."

6. The absorption spectrum of solutions of pure chlorophyll is that of the fresh leaf extract.

Stokes, in 1864, first noticed spectrographically that chlorophyll was a mixture of two components which he called *a* and *b*. He attempted to separate them by means of a differential solubility in alcohol and carbon bisulfide. Willstätter used methyl alcohol and petroleum ether; chlorophyll *a* goes into the petroleum ether and chlorophyll *b* into the methyl alcohol.

Chlorophyll *a* gives a pure yellow phase in a methyl alcoholic solution of potassium hydroxide, and gives only " phytochlorine *e* " as a decomposition product. Chlorophyll *a* crystallizes in thin lance-like leaflets with a blue-steel luster, M. P. 117°–120°. The solutions in ethyl alcohol are bluish-green with a deep red fluorescence.

Chlorophyll *b* gives a dark red phase with a methyl alcohol solution of potassium hydroxide and " phytorhodin *g* " as a decomposition product. The alcoholic solution has a yellow tinge as compared with chlorophyll *a*. Chlorophyll *b* is completely insoluble in cold petroleum ether.

In general, the ratio of occurrence is about 1 molecule of chlorophyll

[6] Schertz, F. M., The Quantitative Determination of Chlorophyll, *Plant Physiol.*, 3 : 323–334 (1928).

[7] Schertz, F. M., The Preparation of Chlorophyll, *Plant Physiol.*, 3 : 487–497 (1928).

b to 3 molecules of chlorophyll *a*. Willstätter has ascribed to chlorophyll *a* the formula, $C_{55}H_{72}O_5N_4Mg + \frac{1}{2} H_2O$, and to chlorophyll *b* the formula, $C_{55}H_{70}O_6N_4Mg$. Adams,[8] on the basis of thermodynamical reasoning, suggests that the formula for chlorophyll *b* may be

$$C_{56}H_{72}O_6N_4Mg,$$

thus differing from chlorophyll *a* by the unit, CO. He notes that the suggested formula is within the experimental error of an elementary analysis.

Adams then suggests the following series of reactions and interactions as accounting for the energy exchanges in photosynthesis: "The chlorophyll may be pictured as going through a cycle of four reactions, two of them associated with the absorption of two quanta each of radiation, and two follow-reactions requiring water and carbon dioxide, but not light:

R represents ($C_{55}H_{72}O_4N_4Mg$)

$\therefore$ R—O is *a*-Chlorophyll; R—CO_2 is *b*-Chlorophyll

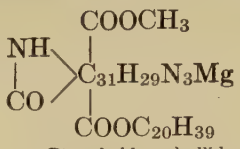
$$\begin{aligned} &R—O + H_2O + 2 \text{ Quanta } \lambda\ 666\ m\mu \rightarrow R + H_2O_2 \\ &R + CO_2 \rightarrow R—CO_2 \\ &R—CO_2 + H_2O + 2 \text{ Quanta } \lambda\ 640\ m\mu \rightarrow R—CO + H_2O_2 \\ &R—CO + H_2O \rightarrow R—O + HCHO." \end{aligned}$$

Chlorophyll may be obtained in both amorphous and in crystalline form. The Russian botanist, Borodin, discovered crystalline chlorophyll in 1881. Willstätter was unable to isolate crystalline chlorophyll in experiments dealing with approximately 200 varieties of plants. He believes that it does not occur in plants but that in the process of isolation the amorphous chlorophyll loses its phytol by action of the enzyme, *chlorophyllase* (which is more or less active in alcoholic media), and the chlorophyll becomes esterified instead, with the alcohol which was used as a solvent, thus causing alcoholysis of the amorphous chlorophyll, and this derived chlorophyll product is the crystalline variety, *i.e.*, amorphous chlorophyll is phytol chlorophyllide and crystalline chlorophyll is methyl or ethyl chlorophyllide.

The parent substance of chlorophyll is a tricarboxylic acid which Willstätter calls *chlorophylline*, $C_{31}H_{29}N_4Mg(COOH)_3$. In chlorophyll one carboxyl group is present as a methyl ester, one as the ester of phytol

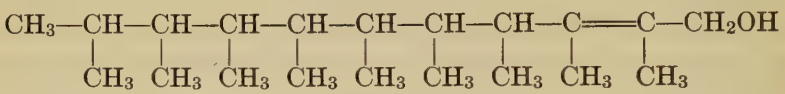
[8] Adams, E. Q., The Efficiency of Photosynthesis by *Chlorella*, *J. Am. Chem. Soc.*, 48 : 292–294 (1926).

(an aliphatic alcohol of formula, $C_{20}H_{39}OH$), and a third is probably present in a *lactam* ring, thus giving a formula of

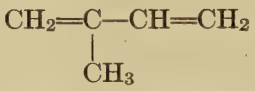


Phytol chlorophyllide *a*
(Amorphous chlorophyll)

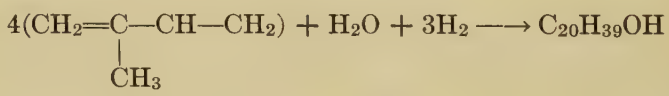
Phytol is an unsaturated aliphatic alcohol with many side chains, presumably mostly methyl groups. The following formula is accepted provisionally,



Isoprene, the hydrocarbon of rubber and terpenes, has the formula,



It has been suggested that the mode of formation of phytol may be the condensation of 4 molecules of isoprene with 1 molecule of water, followed by a reduction.

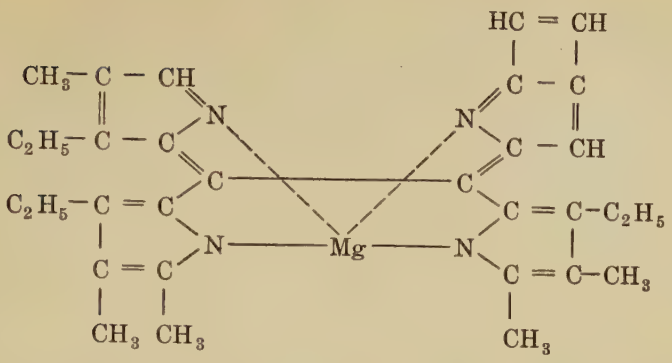


Chlorophyll *a* apparently contains a lactam group which appears to be lacking in chlorophyll *b*. Chlorophyll *b* should contain one carboxyl group more than chlorophyll *a* in order to explain certain of its decomposition products.

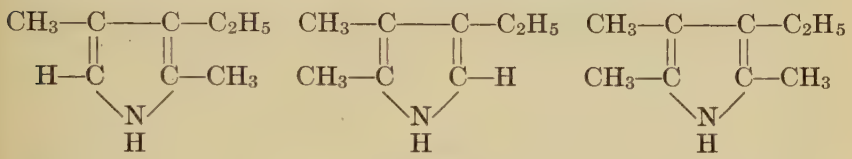
Phytochlorine *e*, the decomposition product of chlorophyll *a* is a tricarboxylic acid with two free carboxyl groups and one bound as a lactam.

Phytorhodin *g*, is a tetracarboxylic acid, one or two of the acid groups being bound, but the exact mode of combination is still uncertain.

It is impracticable to discuss in detail the various decomposition products of the chlorophylls. Adequate references have already been indicated. The groupings of the nucleus have, however, been identified and have a very important biological bearing. Willstätter pictures the nucleus of chlorophyll, *aetiophyllin*, as $C_{31}H_{34}N_4Mg$.



The reduction of chlorophyll yields certain nitrogen compounds, substituted pyrrole rings. The following have been identified:



(1) Kryptopyrrole (2) Isohemopyrrole (3) Phyllopyrrole

(1) *and* (2) *are components derived both from hemin, the red pigment of hemoglobin and from chlorophyll.* Insofar as the author is aware, (3) has been isolated only from chlorophyll (*i.e.*, has not as yet been identified as a decomposition product of the blood pigment).

If one compares the formula of aetiophyllin with the formulae which have been suggested for hemin (Chapter XVI), it will be observed that there is a very striking similarity in the structure of these two pigments, the one the vital pigment of the autotrophic plants, the other the vital pigment of most representatives of the animal kingdom. It is highly improbable that the close similarity in the structure of these two vital pigments is one of chance, but it appears more probable that in the processes involved in organic evolution, the essential nucleus of the earlier vital pigment, chlorophyll, became modified so as to assume new functions in the developing animal kingdom, the *magnesium* in the chlorophyll being replaced with *iron* (or *copper* in the hemocyanin of the *crustaceae*, or *manganese* in the pinnaglobin of *Pinna squamosa*) in order to care for the new function as an oxygen carrier, the branched-chain aliphatic alcohol, phytol, being similarly replaced by a protein residue (a histone, globin), possibly because the animal body cannot synthesize such compounds as phytol but can reconstruct a protein molecule from the amino acids which are secured from the food.

The above hypothesis is strengthened by the observation of Küster,[9] who suggests that hemoglobin is in reality a mixture of two compounds, hemoglobin *a* and hemoglobin *b*, differing only in the fact that one has a free carboxyl group, whereas in the other this carboxyl group is internally linked within the molecule, thus affording an even closer parallelism to the chemical structure of hemoglobin and chlorophyll.

Both chlorophyll and hemin yield " porphyrins " when acted upon by acids. These porphyrins are magnesium- and iron-free, respectively. Chlorophyll *a* and chlorophyll *b* yield the same porphyrins, and *these are isomeric with those formed from hemin.*

Bürgi[10] suggests a stimulatory effect from eating chlorophyll, stating that chlorophyll facilitates the production of hemoglobin.

Magnesium is an integral constituent of the chlorophyll molecule. Prior to the demonstration of magnesium in chlorophyll, it was well known that magnesium was an essential plant food, but the presence of magnesium in chlorophyll was the first demonstrated function of the element. Apparently it should be as essential as is either phosphorus or potash, yet agricultural experiment stations have paid practically no attention to the possibilities of a magnesium hunger or magnesium fertilization. In chlorosis, magnesium may enter as well as other factors. Garner[11, 12] and his coworkers have reported a magnesium deficient soil.

Schertz[13] notes that in the United States alone more than 6,000,000 tons of chlorophyll are produced each year by the corn and small grain crops. He also notes that it possesses some commercial importance and that, in 1924, 3213 pounds of chlorophyll, valued at $5799, were imported into the United States to be used in medicines and as coloring for candles, waxes, resins, oils, soaps, foods, etc.

Carotinoids.—Palmer[14] has prepared a monograph in which the

[9] Küster, W., Individuelle Blutuntersuchungen. IV. Über das Entstehen der Hämine aus dem Hämoglobin A und über die Existenz zweier Hämoglobine A a und A b, *Z. physiol. Chem.*, 151 : 56–85 (1926).

[10] Bürgi, E., Ueber die therapeutische Bedeutung des Chlorophylls, *Deut. med. Wochschr.*, 48 : 1159–1161 (1922).

[11] Garner, W. W., McMurtrey, J. E., and Moss, E. G., Sand Drown, a Chlorosis of Tobacco and Other Plants Resulting from Magnesium Deficiency, *Science*, 56 : 341–342 (1922).

[12] Garner, W. W., McMurtrey, J. E., Bacon, C. W., and Moss, E. G., Sand Drown, a Chlorosis of Tobacco Due to Magnesium Deficiency, and the Relation of Sulfates and Chlorides of Potassium to the Disease, *J. Agr. Res.*, 23 : 27–40 (1923).

[13] Schertz, F. M., Commercial Applications of Chlorophyll Derivatives, *Ind. Eng. Chem.*, 19 : 1152–1153 (1927).

[14] Palmer, L. S., Carotinoids and Related Pigments, American Chemical Society Monograph No. 9, Chemical Catalog Company, Inc., New York (1922). Cf. also

literature of the carotinoid pigments has been exhaustively discussed. Accordingly only incidental reference to some of the outstanding properties of these compounds will be included here.

Palmer notes that the term, carotinoids, should be applied to most of the red, orange, and yellow pigments which can be extracted, by the use of fat solvents, from biological tissues either of plant or of animal origin. In the plants certain of these pigments appear to be invariably associated with chlorophyll in the chloroplasts, and it is only recently that it has become generally recognized that the " lipochromes " of animal tissues are identical with the plant carotinoids.

The carotinoids of the chloroplasts include both *carotin*, an unsaturated hydrocarbon having the formula, $C_{40}H_{56}$, and several pigments included in the group of xanthophylls, which may be designated as *xanthophylls a*, *a'*, *a''*, and β, having the general formula, $C_{40}H_{56}O_2$, and differing from each other chiefly in the position of the absorption bands, although they show somewhat different solubilities and colors in the various solvents.

The carotinoids possess very great tinctorial power, so that a relatively small amount of the actual pigment may produce a relatively deep color. The pigment of carrots appears to be largely carotin. The amount which is present, however, is extremely small. Escher isolated 125 grams of pure carotin from 472 kilograms of dried (5000 kilograms of fresh) carrots.

An isomer of carotin, *lycopin*, is the red pigment of the ripe tomato fruit. Lycopin shows somewhat different solubilities from those of carotin and has somewhat different absorption spectra. Carotin, in small amounts, accompanies lycopin in the tomato.

The brown algae are characterized by the presence of still another carotinoid, *fucoxanthin*, which has the empirical formula, $C_{40}H_{56}O_6$. The red algae are pigmented with phycoerythrin which, as already noted, is not a carotinoid but a protein. The green algae contain both carotin and xanthophyll.

Palmer and Eckles have been largely responsible for demonstrating

Schertz, F. M., The Quantitative Determination of Carotin by Means of the Spectrophotometer and the Colorimeter, *J. Agr. Res.*, 26 : 383–400 (1923); The Quantitative Determination of Xanthophyll by Means of the Spectrophotometer and the Colorimeter, *J. Agr. Res.*, 30 : 253–261 (1925); Some Physical and Chemical Properties of Carotin and the Preparation of the Pure Pigment, *J. Agr. Res.*, 30: 469–474 (1925); Some Physical and Chemical Properties of Xanthophyll and the Preparation of the Pure Pigment, *J. Agr. Res.*, 30 : 575–585 (1925); The Extraction and Separation of Chlorophyll ($\alpha + \beta$), Carotin and Xanthophyll in Fresh Green Leaves, Preliminary to Their Quantitative Determination, *Plant Physiol.*, 3 : 211–216 (1928).

that the so-called lypochromes of animal tissues are in reality carotinoids which originated in the food and have been deposited in the fat and adipose tissues of the animal. Thus, they have shown that the yellow pigment of butterfat is practically pure carotin. Both carotin and xanthophylls are present in the fat of human milk. Carotin, admixed with some xanthophylls, was found by Palmer and Eckles to be the pigment of the pigmented adipose tissue of cattle, whereas the pigment of the yolk of hens' eggs is almost exclusively xanthophyll.

Palmer, and Palmer and Eckles have demonstrated that the carotin content of the cow's tissues, as well as that secreted in the milk fat, is determined by the carotin content of the ration. When foods rich in xanthophyll, such as yellow maize, were fed, no appreciable pigmentation of the animal tissues or of the milk secretion took place, indicating that the xanthophyll in the food was not transported unchanged through the blood stream to the various tissues, in contrast to the behavior of carotin. Similar experiments by Palmer, and Palmer and Kempster showed that xanthophyll-rich rations increased the pigmentation of egg yolk, whereas carotinoid-poor rations or rations in which carotin was the only carotinoid present caused the production of cartinoid-free egg yolks. The xanthophyll-rich rations produced a rapid coloration in all parts of the body of the white leghorn fowl, whereas carotin-containing foods had practically no effect upon the coloration of the bird's tissues. In the experiments by Palmer and Kempster it was conclusively shown that carotinoids play no role in animal physiology, that they are simply stored in the tissues because they are fat-soluble and are present in the diet. Palmer and Kempster were able to rear chickens to maturity on diets which were devoid of the carotinoids that are normally present in the adipose tissue of fowls. The eggs of such chickens possessed carotinoid-free yolks, but this fact did not impair their fertility, nor were the young chicks hatched from such eggs, inferior to those hatched from normally pigmented eggs. Thus, the white leghorn fowl at least does not require carotinoid pigments for growth, maintenance, or reproduction. It seems probable that this is generally true insofar as the animal kingdom is concerned.

There is no general agreement as to the function of the carotinoids in plants. The fact that they invariably occur associated with chlorophyll in the chloroplasts would permit the assumption that they play some role in the photosynthetic process. They show characteristic absorption bands which would permit of their absorbing light of certain wave lengths and converting this energy to chemical uses. This may be their more important function; or it may be that they act as an oxidation $\leftrightarrows$ reduction system and are concerned with oxygen transference or

oxygen assimilation. Willstätter and Stoll suggest that one possible function of the carotinoids may be to control the equilibrium between chlorophyll *a* and chlorophyll *b*.

The isolation of the carotinoids is attended with certain difficulties due to the great ease with which these pigments are oxidized. All evaporations of solvents should be made *in vacuo* at a low temperature. Since the carotinoids are invariably associated with fats and are soluble only in fat solvents, the method generally employed is to saponify the fats in alcoholic sodium hydroxide and to extract the carotinoids along with the "unsaponified residue." The unsaponified residue of fats usually consists almost entirely of a mixture of sterols which can be removed by precipitating the sterols with digitonin. Following the separation of the sterols, the pigments are recrystallized from appropriate solvents.

The preparation of fucoxanthin is somewhat more difficult than is the preparation of the other carotinoids, due to the fact that the saponification process cannot be used, inasmuch as fucoxanthin reacts with the alkali.

Carotin forms colored solutions in the various fat solvents, as well as in the fatty oils or fatty acids. The carbon bisulfide solutions are characterized by a red-orange to blood-red color. The solutions in other solvents range from a yellow to a golden-yellow. Carotin is unaffected by boiling with alkalies and can be recovered unchanged from such solutions. It is adsorbed from petroleum ether solution by finely divided $HgCl_2$, $CaCl_2$, PbS, etc., but it is not adsorbed from a petroleum ether solution by $CaCO_3$, which fact distinguishes it sharply from the xanthophylls. Tswett made use of this fact in his so-called "chromatographic analysis," in which a petroleum ether or carbon bisulfide solution of the carotinoids is filtered through a column of tightly packed, perfectly dry calcium carbonate. The carotin which is present in the solution passes through unchanged, whereas the xanthophylls are adsorbed by the calcium carbonate. The various xanthophylls show somewhat different intensities of adsorption. Accordingly, the column of calcium carbonate, through which the solution has been filtered, will show a series of pink to rose adsorption bands, the bands being spaced in a manner so as to resemble a series of Liesegang rings. From the number, position, color, and intensity of these adsorption bands, a rough estimate can be obtained as to the number and approximate proportion of xanthophylls present in the solution.

Carotin can be quantitatively removed from 80 to 90 per cent alcohol by shaking with carbon bisulfide or petroleum ether. Conversely, no exchange of pigment takes place when a petroleum ether solution of

carotin is shaken with a solution of 80 to 90 per cent alcohol. This property sharply distinguishes carotin from the xanthophylls and fucoxanthin and affords a means for the separation of carotin from the other carotinoids.

All of the carotinoids in solution in oil or melted fat give a beautiful green color reaction on the addition of a trace of solid ferric chloride, the carotinoids being oxidized with a simultaneous reduction of the iron salt to the ferrous condition.

Relatively little is known in regard to the structural constitution of the carotinoids. Inasmuch as carotin is an unsaturated hydrocarbon, it would be expected to form stable halogen derivatives. Two iodides have been described, one corresponding to the formula, $C_{40}H_{56}I_2$, the other to the formula, $C_{40}H_{56}I_3$. Bromine, however, both adds and substitutes in the carotin molecule, the compound which can be isolated having the formula, $C_{40}H_{36}Br_{22}$.

Xanthophyll might be regarded as an oxidation product of carotin but no one has as yet been able to convert carotin into xanthophyll. It forms an iodide, having the formula, $C_{40}H_{56}O_2I_2$, and a bromide containing no oxygen, having the formula, $C_{40}H_{40}Br_{22}$. Xanthophyll crystals slowly oxidize in the air, resulting in two compounds to which the formulae, $C_{40}H_{56}O_{15}$ and $C_{40}H_{56}O_{18}$, have been ascribed.

Lycopin likewise forms an iodide, $C_{40}H_{56}I_2$, and a bromide, $C_{40}H_{44}Br_{26}$.

Fucoxanthin similarly oxidizes readily to form colorless solutions from which compounds, having the formulae, $C_{40}H_{54}O_{16}$ and $C_{40}H_{54}O_{14}$, have been isolated. Fucoxanthin forms an iodide, but apparently one which contains four atoms of iodine instead of the normal di-iodide characteristic of the other carotinoids. Palmer notes that the bromide of fucoxanthin does not appear to have been prepared.

Carotinoid studies afford a very striking illustration of how a pure science study, which apparently has no practical importance, may, in case of an emergency, be applied for the benefit of mankind.

Prior to the World War the absorption spectra of the carotinoids and of chlorophyll had been exhaustively studied and mapped. During the World War it became necessary for those in charge of the American troops on the western front to devise some means for the detection of enemy troop movements which were being carried out under camouflages so designed as to simulate green foliage. Natural green foliage reflects both red and green light, due to the fact that carotinoids and chlorophyll are both present in the chloroplasts. Inasmuch as the quantity of chlorophyll greatly exceeds the quantity of the carotinoids, the foliage appears to the eye as a more or less pure green, the reds and yel-

lows of the carotinoids being masked. Accordingly it was possible to construct covered roads for the movement of troops, the roads being camouflaged to represent an integral part of the landscape by having the covering painted with green paint. To the eye of an observer in an aeroplane, such camouflaged roads would appear to merge with the green of the surrounding fields.

The Eastman Kodak Company devised a light filter capable of screening out the green rays characteristic of chlorophyll but permitting the wide band in the red at about 700 mμ and a narrow band in the green at about 500 mμ, bands characteristic of the carotinoids, to pass through the filter. Accordingly, when a landscape is viewed through such a filter, the natural vegetation does not appear green but instead as a more or less bright red or orange-red,[15] giving an appearance as if the landscape were on fire. A strip of green paint in such a field still appears green when viewed through such a ray filter, inasmuch as the paint pigment does not possess the characteristic optical properties of chlorophyll. Accordingly, when a landscape was observed, from an aeroplane, to have the appearance of a prairie fire and a thin green line appeared across such a landscape, it was easy to determine exactly where to place the aeroplane bombs.

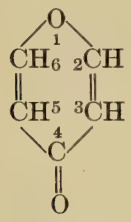
No one could have predicted that research dealing with the absorption spectra of chlorophyll and the carotinoids would ever be turned to practical use, and there is no reason for believing that many of the pure science studies which have been or are being carried out in other fields may not have similar or even greater practical importance.

[15] The summer foliage, viewed through this filter, has the brilliant coloration characteristic of the autumn days.

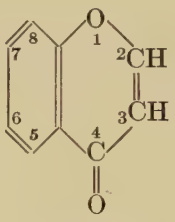
CHAPTER XXX

THE FLAVONES, XANTHONES, ANTHOXANTHONES, AND ANTHOCYANINS

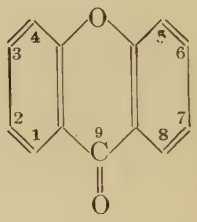
THE FLAVONES (Latin, flavus = yellow), XANTHONES (Greek, $\chi\alpha\nu\theta\sigma\sigma$ = yellow), OR ANTHOXANTHONES.[1]—These are a class of naturally-occurring plant pigments and may be regarded as phenyl derivatives of the 1.4- or γ-pyrone nucleus.



1.4- Pyrone
or
γ-Pyrone

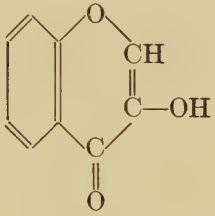


1.4- Benzopyrone or
Benzo-γ-pyrone or
Chromone



Dibenzo-γ-pyrone
or
Xanthone

The keto group in these compounds does not behave as a typical carbonyl group, *e.g.*, the oxygen is not replaceable by hydroxylamine to form oximes. In addition, the ring oxygen is *basic* and adds acid as though it were *quadrivalent* oxygen, resulting in the formation of oxonium salts. The substitution of a hydroxyl group in position (3) in the chromone nucleus forms chromonol.

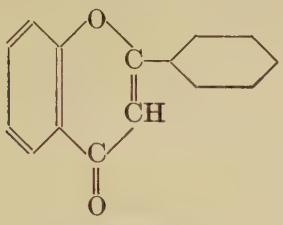


Chromonol

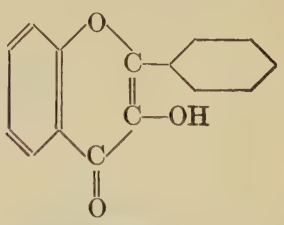
Coloring matters of the chromone and chromonol type have not as

[1] Possibly the term, "anthoxanthones," is best, since this term indicates their similarity to the anthocyanins.

yet been found in nature but the benzene derivatives are the *flavones* or *flavonols*. Almost all of the derivatives of flavonol are yellow dyes.

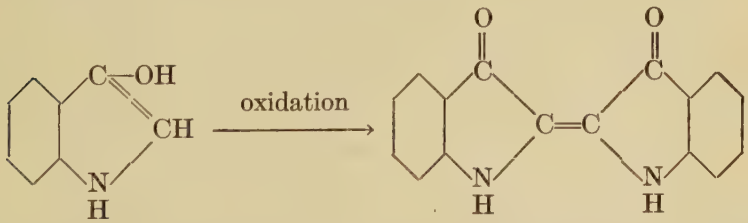


Flavone
(Phenylated Chromone)



Flavonol
(Phenylated Chromonol)

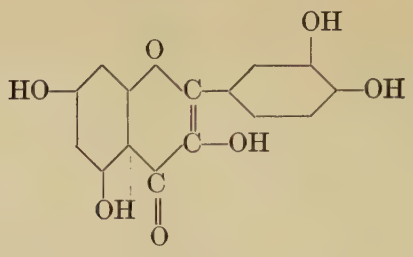
Watson[2] has brought together a considerable part of our knowledge in regard to those groups which produce color in an organic compound and those auxiliary groups which convert a colored compound into a pigment or a dyestuff. Those groups which produce color are called *chromophore* groups. Typical chromophore groupings are the nitro group (—NO_2), the azo group (—N=N—), the quinone grouping (*ortho* or *para* O=C_6H_4=O), etc. Such groups do not necessarily produce pigments or dyestuffs. For example, azobenzene is not a dyestuff. If, however, we add one or more *auxochrome* (intensifying or modifying) groups, we obtain a dyestuff. Typical auxochrome groups are amino groups, hydroxyl groups, sulfonic acid groups, etc. In plants the organic compound containing the auxochrome groups but containing the chromophore group in a reduced or otherwise altered form, is often spoken of as a chromogen. Thus, indoxyl is a colorless *chromogen* which oxidizes to indigo blue or indigotin.



The color of the indigotin can be altered by the introduction of auxochrome groups, *e.g.*, Br, NH_2, NO_2, OH, etc., into the benzene nuclei.

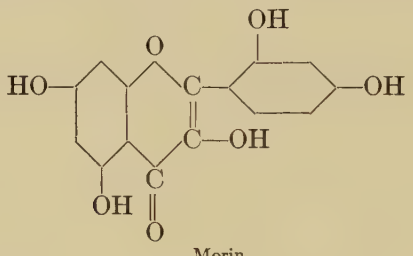
Quercetin, the coloring matter of quercitrin extract, is a tetrahydroxy derivative of flavonol.

[2] Watson, E. R., Colour in Relation to Chemical Constitution, Longmans, Green and Company, London (1918).



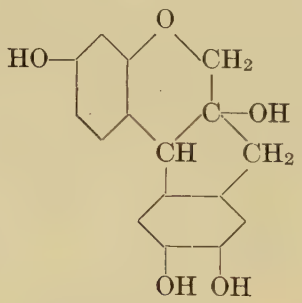
Quercetin

The morin of fustic (Cuba wood) differs from quercetin only in the position of the hydroxyl groups on the benzene nuclei.



Morin

Brazilin, a reduced γ-pyrone derivative, is the coloring principle of hypernic. The constitution was established in 1908 by Sir W. H. Perkin, Jr., who showed it to have the formula,

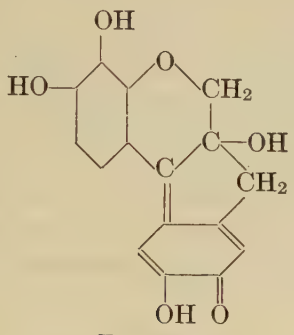


Brazilin

Hematoxylin, from logwood may be regarded as a molecule of brazilin containing one additional hydroxyl group in position (8). Logwood is the source of an excellent black dye. It " weights " silk to about 200 per cent of the original weight of the silk, and Chapin[3] notes that the volume of the silk is enlarged so that the fiber has greater resistance to wear.

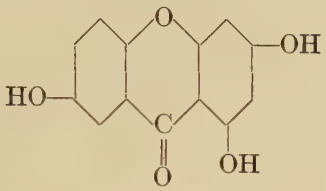
[3] Chapin, E. S., Natural Dyestuffs—An Important Factor in the Dyestuff Situation, *J. Ind. Eng. Chem.*, 10 : 795–798 (1918).

By the oxidation of hematoxylin we obtain hematine. In the oxidation process an additional chromophore grouping (*p*-benzoquinone) has been introduced into the molecule.



Hematine

The xanthone dyestuffs are not important in so far as their natural occurrence is concerned. *Gentisin*, the coloring matter of the gentian root, is a trihydroxyxanthone.



Gentisin

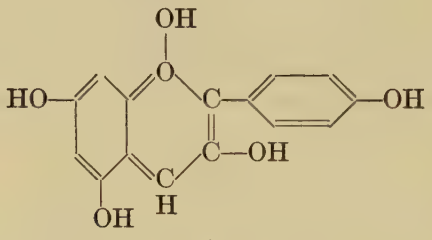
Only one or two other natural pigments belong to the xanthone group.

A great deal of our knowledge of the flavones is due to the work of Perkin and those associated with him (cf. Perkin and Everest[4]). The intensity of the color which this group of compounds possess depends markedly on the position of the hydroxyl groups, and as a rule the color is intensified if two hydroxyl groups are *ortho* in position to each other. This group of pigments usually occurs in plants as the glucosides, one or more hydroxyl groups being combined with a sugar molecule. This has the result that the auxochrome group is rendered inactive so that *in the plant* the flavone glucosides are practically colorless. On hydrolysis of the glucoside, the color develops. Almost all white flowers turn yellow when exposed to the vapors of ammonia, due to a salt formation of ammonium with the flavones (and possibly in part to hydrolysis of glucosides).

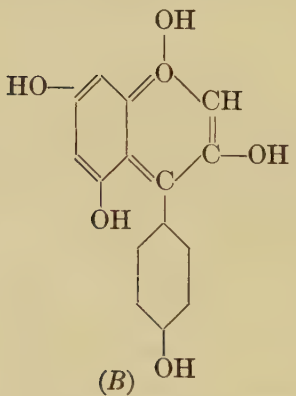
[4] Perkin, A. G., and Everest, A. E., The Natural Organic Colouring Matters, Longmans, Green and Company, London (1918).

As a rule, the flavone type of pigments does not occur in animal tissues. Thomson[5] has, however, isolated a flavone in the study of the pigments of the wings of the butterfly, *Melanargia galatea.* The flavone had the properties of quercetin, although it was not positively identified. In a later paper, Thomson[6] notes that the larva of the butterfly feeds upon the grass, *Dactylis glomerata,* and that this grass contains a flavone-glucoside, the flavone of which is identical with the pigment isolated from the wings of the butterfly. Apparently, therefore, this pigment of the butterfly wings has a plant and not an animal origin.

THE ANTHOCYANINS.—The red, violet, and blue pigments present in the blossoms, fruit, and leaves of many plants belong to the group of the anthocyanins. Almost invariably pigments of the above colors belong in this class.



(*A*)



(*B*)

True anthocyanins are derivatives of the γ-pyran or benzopyranol nucleus and all, or practically all, that have been studied are derivatives

[5] Thomson, D. L., The Pigments of Butterflies' Wings. I. *Melanargia Galatea, Biochem. J.*, 20 : 73–75 (1926).

[6] Thomson, D. L., The Pigments of Butterflies' Wings. II. Occurrence of the Pigment of *Melanargia Galatea* in *Dactylis Glomerata, Biochem. J.*, 20 : 1026–1027 (1926).

(by the introduction of additional hydroxyl or methoxy groups) of the compound possessing formula (*A*), although Nierenstein[7] suggests that the formula may be as shown in (*B*). For the present we will adhere to the older formula (*A*).

The chromophore group in this formula appears to be that of a substituted *ortho*-benzoquinone. The anthocyanins are further characterized by the heterocyclic oxygen functioning as *tetravalent oxygen, i.e.*, the anthocyanin compounds readily add acids to form *oxonium salts.* Solutions of the anthocyanins act as *indicators,* the anthocyanin molecule readily combining with acids to form compounds where the negative radical is bound to the oxygen. All or nearly all anthocyanins are *red* (or purple) in acid solution and possess green or blue shades in neutral or alkaline solution.

The anthocyanins are very widely distributed throughout the plant kingdom, the majority of the higher plants containing anthocyanin at some stage in their development. It is to anthocyanins that we owe the brilliant colors of our flowers, our foliage plants, and our autumn landscapes.

The function of anthocyanins in the plant is a subject which is still open to speculation. They are apparently rather easily reduced and oxidized, and it has been suggested by many, notably Palladin, that they are respiratory pigments and act as oxygen carriers, assisting in cell oxidations and reductions. This, however, still remains to be definitely proven.

The Alpine plants are particularly rich in anthocyanins and lose much of their color when grown at lower levels. One suggestion as to a possible function is that they may serve to screen out the injurious short wave lengths of light which pass through the thin atmosphere at the higher elevations. The pigment also acts as an absorber of the heat rays. Leaves colored with anthocyanins may be as much as 2° C. warmer than other leaves on the same plant which contain only the green pigment and which have the same light exposure.

Of recent years botanists and geneticists have turned to the inheritance of color in plants in order to study the laws governing heredity. Color is easy to see and to follow in the progeny through successive generations; therefore, the inheritance of color has been studied. The early workers did not appear to realize that what they were in reality studying was the *inheritance of chemical compounds and the inheritance of chemical reactions* where more than one component was involved. Miss Whel-

[7] Nierenstein, M., The Constitution of Catechin. IX. Some New Disintegration Products of Acacatechin, *J. Am. Chem. Soc.*, 48 : 1959–1975 (1926).

dale[8-12] (Mrs. Onslow) pointed this out, and later Keeble and Armstrong[13-15] and Keeble, Armstrong, and Jones[16] added further details, so that we now have a fairly good idea of the chemistry of color inheritance in certain flowers.

Mendel's law of inheritance has been found to apply to the inheritance of color in flowers. The only method of learning the genetic factors involved is by crosses between parents which differ in the expression of one or more characters. In crosses between homozygous parents, the first generation offspring is uniform and segregation occurs in the germ cells of this hybrid. The second generation is therefore characterized by different types.

For example, let us assume that (A) represents absence of color, and (B) represents a blue color. If the germ cells of one parent of a cross are all AA, *i.e.*, this individual has received no genetic factor for color from either of its parents, and the germ cells of the other parent of a cross are BB, *i.e.*, this individual has received a genetic factor for blue color from both of its parents, then we can predict the genetic constitution of the F_1 (first generation) and of the F_2 (second generation) offspring, which according to Mendel's law will have a genetic constitution as represented in the diagram on page 623.

The first generation offspring will all possess the blue pigment, inasmuch as each individual contains the factors which are present in both parents. When, however, the first generation offspring are inbred, segregation will occur in the second generation, so that the F_2 offspring may

[8] Wheldale, M., The Chemical Differentiation of Species, *Biochem. J.*, 5 : 445–456 (1911).

[9] Wheldale, M., The Flower Pigments of *Antirrhinum Majus*. I. Method of Preparation, *Biochem. J.*, 7 : 87–91 (1913).

[10] Wheldale, M., and Bassett, H. L., The Flower Pigments of *Antirrhinum Majus*. II. The Pale Yellow or Ivory Pigment. III. The Red and Magenta Pigments, *Biochem. J.*, 7 : 441–444 (1913); 8 : 204–208 (1914).

[11] Wheldale, M., and Bassett, H. L., The Chemical Interpretation of Some Mendelian Factors for Flower-Colour, *Proc. Roy. Soc.*, B., 87 : 300–311 (1914).

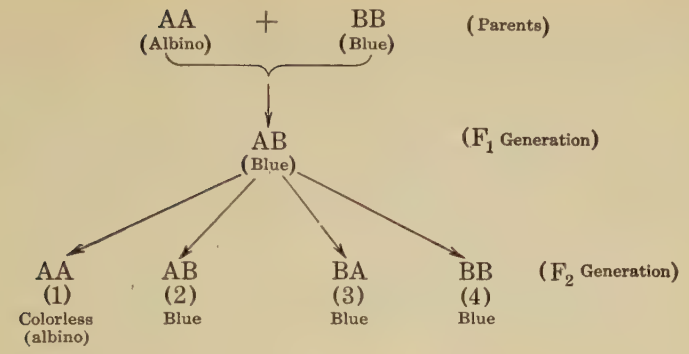
[12] Wheldale, M., Our Present Knowledge of the Chemistry of the Mendelian Factors for Flower-Colour, Pt. I and Pt. II, *J. of Genetics*, 4 : 109–129; 369–376 (1914 and 1915).

[13] Keeble, F., and Armstrong, E. F., The Oxydases of *Cytisus Adami*, *Proc. Roy. Soc.*, B., 85 : 460–465 (1912).

[14] Keeble, F., and Armstrong, E. F., The Distribution of Oxydases in Plants and Their Role in the Formation of Pigments, *Proc. Roy. Soc.*, B, 85 : 214–218 (1912).

[15] Keeble, F., and Armstrong, E. F., The Role of Oxydases in the Formation of the Anthocyan Pigments of Plants, *J. of Genetics*, 2 : 277–311 (1912).

[16] Keeble, F., Armstrong, E. F., and Jones, W. N., The Formation of the Anthocyan Pigments of Plants, Pt. VI, *Proc. Roy. Soc.*, B, 87 : 113–131 (1913).



possess any one of four possible genetic constitutions. (1) and (4) will breed true. Such individuals are called homozygous, *i.e.*, all the germ cells which they produce possess the same genetic constitution for the factor in question. The progeny produced by the self-fertilization of (1) will always be colorless, and the progeny produced by the self-fertilization of (4) will always be blue. (2) and (3) are heterozygous, *i.e.*, they produce germ cells which do not have the same genetic constitution for the factor in question, so that when AB or BA are inbred resegregation will occur in the third generation to give a ratio of one colorless to four blue offspring, having respectively the genetic constitution of AA, AB, BA, and BB.

(5) (6) (7) (8)

Again (5) and (8) will be homozygous and will breed true, and (6) and (7) will again be heterozygous and give one colorless (albino), and three blue, only one of which (BB) will breed true.

Now assuming that color is the resultant of two factors, *e.g.*, an oxidizable chromogen (C) and an oxidizing enzyme (E), in order to form color, both (C) and (E) must be present. We can thus postulate white flowers which have the following genetic formulae:

(1) White = AA = albino (contains neither chromogen nor oxidase).
(2) White = CC (contains only chromogen).
(3) White = EE (contains only enzyme).

The following matings will produce

AA × CC = white offspring (only one factor for color is present).
AA × EE = white offspring (only one factor for color is present).
CC × EE = *colored* offspring because *both* factors for color, enzyme and chromogen, are present.

Such a postulate may be used to explain why certain white flowers

crossed with other white flowers may yield color, whereas the same flowers self-fertilized yield only white offspring.

Still another form of white is known. *Here there is an inhibitor present, some chemical reaction which prevents the formation of pigment when both enzyme and chromogen are present.* Such a white is known as a *dominant white* and when crossed with a colored variety, results in white offspring. Thus, the white leghorn fowl is a *dominant white,* and yet it is not an albino. The white " Silky " fowl insofar as the feathers are concerned is an albino (*recessive white*).

The animal pigment, melanin, is formed by the interaction of a chromogen and an oxidase. The oxidizing enzyme is apparently tyrosinase (cf. Gortner[17-19]). Gortner[20] found that *m*-dihydroxyphenols (orcinol, resorcinol, and phloroglucinol) inhibit the oxidation of tyrosine by tyrosinase, yet do not destroy the tyrosinase, nor are they themselves oxidized in preference. Apparently their presence is sufficient to inhibit the action. While no similar chemical has as yet been isolated from a dominant white organism, this test tube experiment is at least suggestive of the mechanism which may be involved.

With the anthocyanins we do not always have a simple genetic formula, *i.e.,* a one factor difference. Studies of the inheritance of color in the sweet pea have shown interesting results. The original wild sweet pea was in all probability a chocolate and purplish-blue flower. By breeding, various colors have been selected, and these can be recombined to the original color. Two whites may give purple, as already noted. This occurs in certain crosses of sweet pea varieties. Red color is therefore due to two factors, A and B, and the loss of either produces a white flower. A third factor, R, is necessary to produce the blue color which when combined with red produces *purple,* but *R has no color when alone,* only when combined with A and B. Thus, flowers containing only A, B, AR, BR, or R are all colorless. Flowers containing AB are red, and flowers containing ABR are purple. From such data, *deductions* as to the *chemical* factors which are involved have been drawn.

Miss Wheldale (Mrs. Onslow) proposed a chemical interpretation of the function of factors for flower color in *Antirrhinum.* The factorial

[17] Gortner, R. A., Studies on Melanin. II. The Pigmentation of the Adult Periodical Cicada (*Tibicen septendecim* L.), *J. Biol. Chem.,* 10 : 89–94 (1911).

[18] Gortner, R. A., On Melanin, *Biochem. Bull.,* 1 : 207–215 (1911).

[19] Gortner, R. A., Studies on Melanin. IV. The Origin of the Pigment and the Color Pattern in the Elytra of the Colorado Potato Beetle (*Leptinotarsa decemlineata* Say), *Am. Naturalist,* 45 : 743–755 (1911).

[20] Gortner, R. A., Studies on Melanin. III. The Inhibitory Action of Certain Phenolic Substances upon Tyrosinase. A Suggestion as to the Cause of Dominant and Recessive Whites, *J. Biol. Chem.,* 10 : 113–122 (1911).

composition of the type of this species may be expressed with symbols as follows:

YYIILLTTDDBB

in which the symbols represent the following factors:

Y = a factor representing yellow color in the lips with ivory tube color; the absence of Y precludes the formation of any color in the flower.

I = a factor causing the production of ivory color in the tube and lips and inhibiting the formation of yellow pigment in the lips except on the palate.

L = a factor causing a magenta color in the lips.

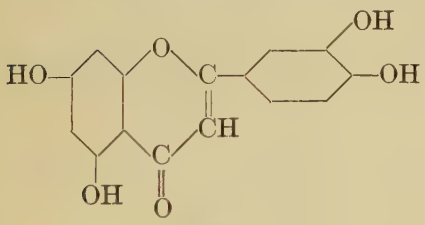
T = a factor causing a magenta color in the tube.

D = a factor causing the production of a deepened or full color, changes a tinged ivory to magenta.

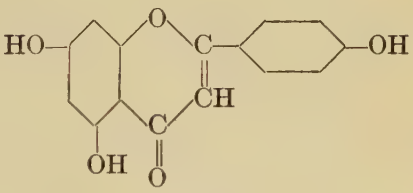
B = a factor converting a red anthocyanin to a magenta anthocyanin.

A study of the chemistry of the pigments involved and the mechanism of color production in *Antirrhinum* makes it possible to interpret these factors in chemical language. Thus, on a chemical basis they are:

Y = a factor causing the production of *Luteolin* in the lips and of *Apigenin* in the tube.



Luteolin



Apigenin

I = a factor causing the production of *apigenin* in the lips in place of luteolin (note that these two compounds differ only by one hydroxyl group).

L = a factor representing the presence of a reducing (?) or an oxidizing agent, enzyme (?), in the lips, which will produce an anthocyanin from the flavones when they are present.

T = a similar factor representing the presence of a reducing (?) or oxidizing (?) agent in the tube.

D = a factor, emulsin (?), causing the splitting off of the sugars from chains of linked glucosides and the addition or rearrangement of hydroxyl and methoxy groups attached to the rings of the

flavone nucleus, thus causing additional anthocyanin to be formed by the hydrolysis of flavone glucosides.

B = a factor controlling the reaction (hydrogen ion concentration) of the cell sap, thus shifting the color of a solution of the anthocyanin from red to magenta.

The following examples, which have been worked out for *Antirrhinum* varieties, show how these factors work:[21]

1. yyIILLTTDDBB "white," lips and tube of corolla are pure white (no *flavone* is produced).
2. YYiillTTDDBB "yellow," lips yellow, tube ivory [luteolin present but no oxidizing (or reducing) agent].
3. YYiiLLTTddbb "yellow tinged with bronze," [luteolin present, oxidizing (or reducing) agent present].
4. YYIIllTTDDBB "ivory," lips and tube ivory [luteolin absent, apigenin present, no oxidizing (or reducing) agent in lips].
5. YYiiLLTTDDbb "bronze," [luteolin absent, apigenin present in lips, glucoside-splitting enzyme (?) present].
6. YYiiLLTTDDBB "crimson," lips crimson, tube magenta, luteolin present in lips and tube, glucoside-splitting enzyme present, hydrogen ion factor present, apigenin absent.
7. YYIILLTTDDBB "magenta," all factors present producing deepest color.

We thus have hereditary factors which can be expressed by

1. The synthesis of definite chemical substances.
2. The modification of such substances once they are formed.
3. The control of enzyme action.
4. The modification of cell sap reaction.

The above discussion may be considered as only a suggestion of the possible aid which biochemistry may furnish in bringing about a solution of the problems of heredity. Only a bare beginning has been made in this important and fruitful field.

When anthocyanins are present, there is, as a rule, an excess of sugars in the leaves. Overton, in 1899, first called attention to the fact that there was more sugar in the red autumn leaves than in the normal

[21] See Wheldale, *J. Genetics*, Vol. 4, color plate VII (1914), for an exact reproduction of these varieties.

green leaves. This has been confirmed by other workers who have also found more glucosides in the leaves containing anthocyanins. This is especially true for plants where the conducting systems have been injured so that the transport of carbohydrates is interfered with. A leaf which is injured may rapidly turn red, while the remainder of the plant has its full vigor. Since anthocyanin chromogens usually occur as glucosides and since the flavone nucleus probably comes originally from a carbohydrate source, it appears logical that an increased carbohydrate content should lead to the formation of excess chromogen which sooner or later is converted into an anthocyanin.

The artificial " feeding " of plants with sugars has resulted in many instances in anthocyanin formation. More intense light and low temperature produce anthocyanin, as has already been noted as a characteristic of alpine plants. Here we have high photosynthetic activity and a low requirement of carbohydrate utilization for energy (low enzyme activity for respiration, due to low temperature) both conducive to the formation of carbohydrates.

Mrs. Onslow[22] believes that chromogens acted on by oxidases produce anthocyanins, and she has produced good *genetical* evidence for her theory. Keeble and Armstrong support her view. Everest, however, in his chemical studies, finds that flavones, when reduced, give colors corresponding to anthocyanins, and regards the anthocyanins as reduction products of the flavones. He has the greater amount of chemical evidence on his side, the only question being as to whether the chemical reagents which are used to isolate the pigments have altered their nature (cf. Wheldale and Bassett[23]). It appears at the present time as though Willstätter's formulae and the *reduction* theory are the most probable, although the genetic data and certain of the biochemical data are somewhat against them (cf. also Wheldale[24, 25]).

The biochemical data are very well summed up by Armstrong,[26] as follows: " In general the distribution of pigments in flowers coincides exactly with that of oxydases. The oxydases, it is true, are more widely distributed than are the chromogens; but the distribution is in con-

[22] Onslow, M. Wheldale, The Anthocyanin Pigments of Plants, Second Edition, Revised, The University Press, Cambridge (1925).

[23] Wheldale, M., and Bassett, H. L., On a Supposed Synthesis of Anthocyanin, *J. Genetics*, 4 : 103–107 (1914).

[24] Wheldale, M., On the Nature of Anthocyanin, *Proc. Cambridge Phil. Soc.*, 15 : 137–168 (1909).

[25] Wheldale, M., On the Formation of Anthocyanin, *J. Genetics*, 1 : 133–158 (1911).

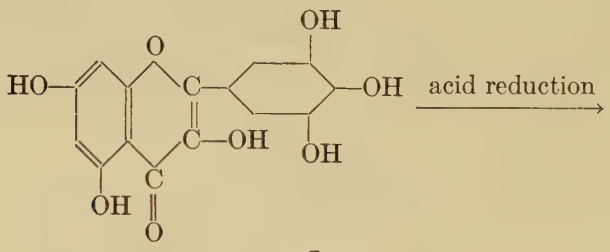
[26] Armstrong, E. F., Chemistry in the Twentieth Century, E. Benn, Ltd., London (1924).

formity with the oxydase-chromogen hypothesis, as will be illustrated by several typical examples, culled from the many available.

" The flowers of *Primula sinensis* and of *Dianthus barbatus* (Sweet William) show most epidermal oxydase in the most deeply coloured varieties, less in the less deeply coloured, and none at all in the white varieties. The white flowers of certain *Primula sinensis*, *Pisum sativum*, and *Lathyrus odoratus* have all been shown to contain oxydases, and the white colour is attributed to the absence of chromogen. In the Mont Blanc Star, the distribution of oxydase again parallels that of pigment. One flower had irregular magenta flaked petals with one exception, this particular petal being of a uniform magenta colour. The latter petal gave a well-marked oxydase reaction, the magenta patches on the others demonstrated a fair reaction, whilst the white portions did not respond to the test.

" Similarly, Sweet Williams were grown in full-coloured, white, and almost white varieties, the latter showing rosy dots or lines. The fully-coloured flowers responded definitely to the tests for the presence of oxydases, whilst the white flowers also gave a definite but limited reaction—the white colour being probably due, as explained above, to the absence of chromogen. The white flowers with rosy dots showed oxydases only in the parts of the petals corresponding to the pigmented dots."

Everest considers that the flavones are converted into anthocyanins by the following reactions:



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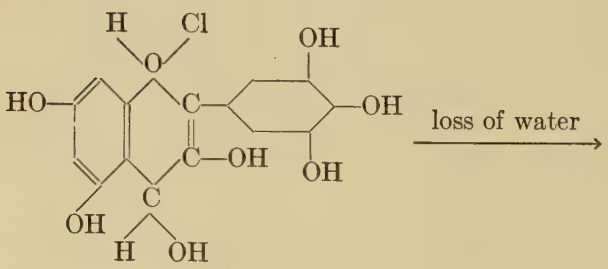
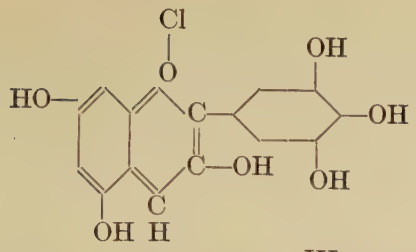


II



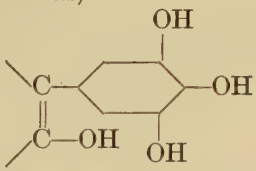
III

Compound II is a colorless or faintly colored intermediate compound. It should be noted that the characteristic carbonyl group of the flavonol nucleus has been reduced and that the orthoquinone grouping is not present, although the tetravalent nature of the ring oxygen still persists. When the elements of water have been lost, a rearrangement takes places, resulting in the formation of the orthoquinone grouping with the resulting color formation.

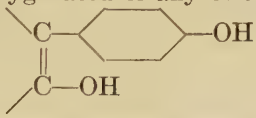
The compound having Formula III is *delphinidin chloride.* Delphinidin is a violet to blue-black anthocyanin, obtained from grapes, hollyhocks, petunias, violets, etc., as glucoside compounds or ethers. The violet, *Viola tricolor*, contains this pigment as a rhamnoglucoside.

Inasmuch as all of the anthocyanins may be regarded as substitution products of the same nucleus, it is unnecessary to repeat all of the groupings in order to show the structural formula of the various anthocyanins.

Representing delphinidin as,

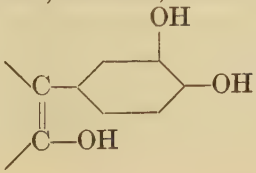


pelargonidin, the least oxygenated of any of the anthocyanins becomes

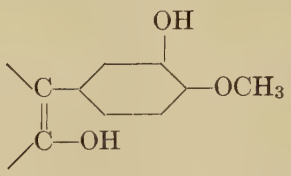


Pelargonidin occurs in many scarlet flowers, scarlet salvia, purple-red asters, etc.

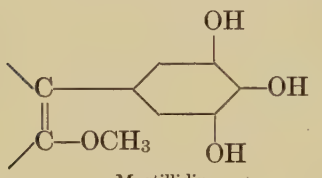
Cyanidin, the anthocyanin of deep red dahlias, cornflower, poppies, fruits of cherries, cranberries, currants, mountain ash, etc., contains the grouping,



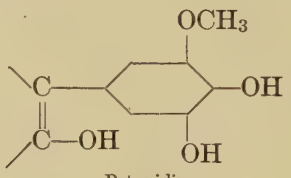
The anthocyanin colors are modified and the complexity of researches in this field are increased by the presence of ethoxy or methoxy groups in the molecule. Thus, *peonidin* is cyanidin monomethyl ether. Peonidin occurs in the deep violet-red peony, as a diglucoside. The problem of



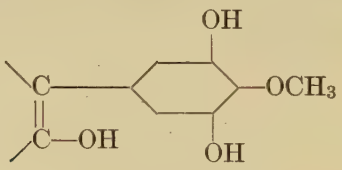
determining the formulae of mono- and dimethyl ethers of an anthocyanin is exceedingly difficult. Taking delphinidin as an example, there are five possible monomethyl ethers. Four have been found in nature. Formulae have been assigned to three of these.



Myrtillidin



Petunidin



Ampelopsidin

There are eleven possible dimethylethers. Only two have yet been isolated and the formulae ascribed to these are only tentative.

Anderson et al.,[27-29] find that an identical dark blue anthocyanin can be isolated from the skins of American grapes, *Vitis labrusca* (Concord), *Vitis aestivalis* (Norton), and *Vitis riparia* (Clinton), and that the com-

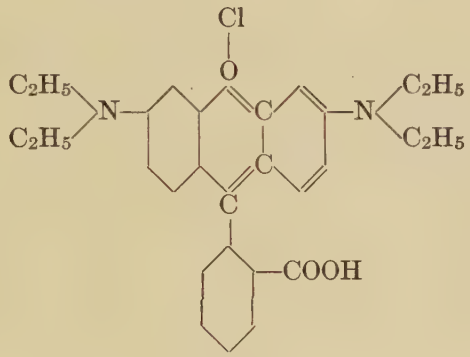
[27] Anderson, R. J., and Nabenhauer, F. P., A Contribution to the Chemistry of Grape Pigments. IV. The Anthocyans in Isabella Grapes, *J. Am. Chem. Soc.*, 48 : 2997–3003 (1926).

[28] Anderson, R. J., Concerning the Anthocyans in Norton and Concord Grapes. A Contribution to the Chemistry of Grape Pigments, *J. Biol. Chem.*, 57 : 795–813 (1923).

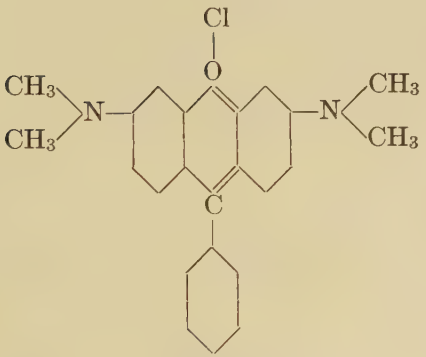
[29] Anderson, R. J., and Nabenhauer, F. P., A Contribution to the Chemistry of Grape Pigments. II. Concerning the Anthocyans in Clinton Grapes, *J. Biol. Chem.*, 61 : 97–107 (1924).

pound is a monomethyl ether of delphinidin. The skin of the European dark blue grape, *Vitis vinifera*, contains the anthocyanin, oenidin, a dimethyl ether of delphinidin. Anderson[30] found that the hybrid grapes produced by crossing the American and European forms, are characterized by the presence of oenidin, the dimethoxy derivative. The tendency to produce a dimethoxy derivative of delphinidin is therefore a dominant in inheritance over the tendency to produce the monomethoxy derivative.

A number of our synthetic " coal tar " dyes are closely allied to the anthocyanin compounds, *e.g.*,



"Rhodamine B"



Tetramethylrosamine

[30] Anderson, R. J., A Contribution to the Chemistry of Grape Pigments. III. Concerning the Anthocyans in Seibel Grapes, *J. Biol. Chem.*, 61 : 685–694 (1924).

CHAPTER XXXI

THE CLASSIFICATION OF THE FATS AND ALLIED SUBSTANCES AND THE GENERAL PROPERTIES OF THE FATS AND OILS

The natural fats and oils may be divided into two major groups, (1) the fatty or non-volatile fats and oils, and (2) the essential or volatile oils. Chemically the two groups are entirely distinct, group (1) being composed of esters of fatty acids (mainly glycerol esters), and group (2) being those plant products volatile with steam and separating as an oily layer in the distillate. Chemically the essential oils are aldehydes, alcohols, acids, hydrocarbons, terpenes, etc. Rarely are they the esters of the ordinary fatty acids. Oil of cloves, wintergreen, turpentine, etc., are typical examples. Because of the great difference in chemical nature, the essential oils are not included in the term, "fats and allied substances."

True fats are composed, in general, of only carbon, hydrogen, and oxygen, but certain derivatives or fat-like compounds, the "lipides," "phospholipides," "lecithins," etc., contain nitrogen or phosphorus or both. A rough classification of the substances in this group may be made as follows:[1]

I. Neutral fats and fatty acids, fatty acid esters of the triatomic alcohol, glycerol.

II. Fatty acid esters of aliphatic or aromatic alcohols other than glycerol—the waxes and the esters of the sterols, or the sterols themselves.

III. Substances containing fatty acids, a nitrogen base, and phosphorus—the "phosphatides" (Thudichum), or "phospholipides." Lecithin is the best known example.

IV. Substances containing a fatty acid, a nitrogen base, and a carbohydrate—the "cerebrosides" (Thudichum), or "glucolipides."

[1] There is as yet no general agreement as to classification (cf. "International Union of Pure and Applied Chemistry, 9th Conference Held at The Hague, July 18–24, 1928," *Ind. Eng. Chem., News Edition*, Sept. 10. 1928, pp. 3–4).

A CLASSIFICATION OF THE FATTY ACIDS

(Those which occur commonly are starred.)

I. The saturated fatty acids, acetic acid series, $C_nH_{2n}O_2$, $C_nH_{2n+1}COOH$ (only those in which n is an even number are found in natural fats).

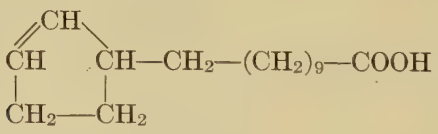
Name	Carbon Atoms	Formula	Occurrence
Acetic	2	$C_2H_4O_2$	Vinegar
Butyric	4	$C_4H_8O_2$	Milk fat
Caproic	6	$C_6H_{12}O_2$	Butter, cocoanut and palm nut oils, etc.
Caprylic	8	$C_8H_{16}O_2$	Cocoanut and palm nut oils, butter, etc.
Capric	10	$C_{10}H_{20}O_2$	Butter, cocoanut and palm nut oils, etc.
*Lauric	12	$C_{12}H_{24}O_2$	Laurel oil, spermaceti, etc.
*Myristic	14	$C_{14}H_{28}O_2$	In nutmeg butter
*Palmitic	16	$C_{16}H_{32}O_2$	Animal and vegetable fats
*Stearic	18	$C_{18}H_{36}O_2$	Animal and vegetable fats
*Arachidic	20	$C_{20}H_{40}O_2$	Peanut Oil
Behenic	22	$C_{22}H_{44}O_2$	Oil of ben, from seeds of *Moringa pterygosperma*
Lignoceric	24	$C_{24}H_{48}O_2$	In glucolipides and arachis oil
Carnaübic	24	$C_{24}H_{48}O_2$	Carnauba wax
Cerotic	26	$C_{26}H_{52}O_2$	Beeswax, Chinese wax, opium wax, wool fat, etc.
Melissic	30	$C_{30}H_{60}O_2$	

II. The unsaturated fatty acids

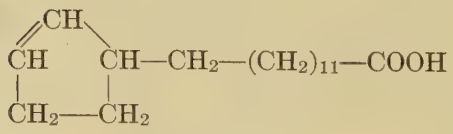
1. Acrylic or oleic acid series	$C_nH_{2n-1}COOH$	
Tiglic	$C_5H_8O_2$	Croton Oil
*Oleic	$C_{18}H_{34}O_2$	Animal and vegetable fats
Eläidic	$C_{18}H_{34}O_2$	Does not occur in natural fats
*Erucic	$C_{22}H_{42}O_2$	Rapeseed and similar oils
2. Linoleic or linolic acid series. Acids with two double bonds; only C_{18} acids occur in nature; the exact position of double bonds is unknown.	$C_nH_{2n-3}COOH$	
*Linoleic	$C_{18}H_{32}O_2$	Vegetable oils, such as linseed, cottonseed, etc.

A CLASSIFICATION OF THE FATTY ACIDS—*Continued*

Name	Formula	Occurrence
Tariric	$C_{18}H_{32}O_2$	
Elomargaric	$C_{18}H_{32}O_2$	Chinese wood oil
3. Linolenic acid series Acids with three double bonds; only the C_{18} acids occur in nature.	$C_nH_{2n-5}COOH$	
*Linolenic	$C_{18}H_{30}O_2$	Linseed oil
4. Series Acids with four double bonds.	$C_nH_{2n-7}COOH$	
*Clupanodonic	$C_{18}H_{28}O_2$	Japanese sardine oil
III. Saturated monohydroxy acids	$C_nH_{2n}O_3$	
α-Hydroxy-*n*-decanic	$C_{10}H_{20}O_3$	Brain phospholipides
Lanopalmic	$C_{16}H_{32}O_3$	Wool fat
Cerebronic or phrenosinic	$C_{25}H_{50}O_3$	Phrenosin (brain tissue)
Cocceric	$C_{31}H_{62}O_3$	Cochineal wax
IV. Unsaturated monohydroxy acids	$C_nH_{2n-2}O_3$	
Ricinoleic	$C_{18}H_{34}O_3$	Castor oil
V. Saturated dihydroxy acids	$C_nH_{2n}O_4$	
Dihydroxystearic	$C_{18}H_{36}O_4$	Castor oil
Lanoceric	$C_{30}H_{60}O_4$	Wool fat
VI. Saturated dibasic acids	$C_nH_{2n-2}O_4$	
Japanic	$C_{22}H_{42}O_4$	Japan wax
VII. Chaulmoogric series Cyclic acids with one double bond		
Hydnocarpic	$C_{16}H_{28}O_2$	Chaulmoogra oil
Chaulmoogric	$C_{18}H_{32}O_2$	Chaulmoogra oil



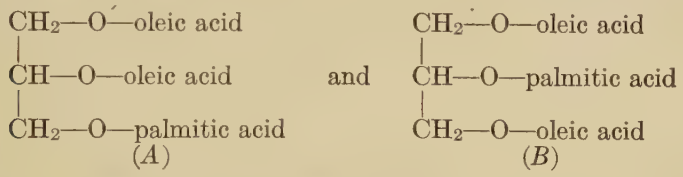
Hydnocarpic acid [2]



Chaulmoogric acid [2]

[2] Noller, C. R., and Adams, R., Synthesis of Dihydrochaulmoogric and Dihydrohydnocarpic acids. II., *J. Am. Chem. Soc.*, 48 : 1080–1089 (1926).

Inasmuch as glycerol is a trihydroxy alcohol, we have possible a number of esters. Thus, for example, we may have monoglycerides, diglycerides, and triglycerides. In the case of the di- and triglycerides we may have the same or different fatty acids united in the ester, resulting in the formation of simple or mixed glycerides. Mixed glycerides appear to be the general rule in nature. The milk fat of the monotreme or Australian spiny ant-eater is a notable exception. Marston[3] notes that the milk fat of this animal appears to be pure triolein. When mixed glycerides occur, the problem of space relationships enters to complicate a study of the chemical constitution of the glyceride. Thus, for example, the two fats,



will have the same empirical formula, will yield the same quantities of the same products upon hydrolysis, but because of the space configuration of the molecule, they will exhibit somewhat different physical properties. For example, (*A*) will be optically active, whereas (*B*) will not. When such facts are kept in mind, together with the fact that the fats are relatively inert chemically and practically always occur as mixtures which are exceedingly difficult to separate, some appreciation can be gained of the difficulties involved in a study of the organic chemistry of the fats and oils.

Mono- and diglycerides apparently never occur in nature, nor are they present in freshly prepared oils and fats. Their presence in a fat or oil indicates that the material has undergone partial saponification. The presence of free fatty acid likewise indicates that hydrolysis of the neutral triglycerides has taken place.

The fatty acids containing ten or less carbon atoms are classified as the *soluble fatty acids*. All fatty acids containing more than ten carbon atoms are classified as insoluble fatty acids, although lauric acid is slightly soluble in boiling water. As would be expected, the solubility of the fatty acids in water decreases as the number of carbon atoms increases. This has already been indicated in the discussion of the phenomena involved in the molecular orientation at interfaces. The *volatile fatty acids* are the same six fatty acids which are classified as soluble fatty acids. These are the only ones which can be distilled at atmos-

[3] Marston, H. R., The Milk of the Monotreme—*Echidna aculeata multi-aculeata, Australian J. Exp. Biol. Med. Sci.*, 3 : 217–220 (1926).

pheric pressure. They possess a fairly high boiling point, but because of the high vapor tension, they can be readily removed from an aqueous solution by steam distillation. Lauric acid is slightly volatile under the same conditions. Most natural fats contain some of the volatile fatty acids.

The titration value of a steam distillate of a saponified fat is the means of determining the *Reichert-Meissl number* of a fat, *i.e.*, the Reichert-Meissl number is the number of cc. of 0.1 N KOH required to neutralize the steam distillate obtained from 5 grams of saponified fats. Butterfat, because of its butyric acid content, has a very high (for fats) Reichert-Meissl number. Jensen[4] notes that this process accounts for 85 to 88 per cent of the total butyric acid, 85 to 100 per cent of the caproic acid, and 24 to 25 per cent of the caprylic acid present in the glycerides of butterfat. From 10 to 13.6 per cent of the fatty acids of butterfat are volatile.

The insoluble fatty acids differ somewhat in chemical structure, which fact permits their classification into the saturated and unsaturated fatty acids, the unsaturated fatty acids being further subdivided according to the type and degree of unsaturation, as has already been noted in the early part of this chapter. Oleic, stearic, and palmitic acids constitute the principal insoluble fatty acids occurring in nature.

The Properties of Natural Fats and Oils.—All the naturally-occurring fats and oils have a specific gravity less than 1.0. Many of the fats can be obtained in a crystalline form. Both fats and oils are readily soluble in the so-called fat solvents, *i.e.*, ether, petroleum ether, chloroform, carbon bisulfide, and carbon tetrachloride, etc., but they are, in general, only sparingly soluble in alcohol, and because of this fact, ethyl alcohol is usually chosen as a solvent from which attempts are made to prepare crystalline fats. The fats themselves, the fatty acids derived from them, and especially the alkali salts of the fatty acids or soaps greatly reduce the surface tension of water. Accordingly, the soaps are used generally as emulsifying agents or detergents. The fats are excellent solvents for other compounds which have similar nature and which have similar solubilities. As we shall have occasion to note later, pure lard is often used as a solvent to gather the essence of flowers in the manufacture of perfumes. An analogous instance is the phenomenon which occurs when onions and butter are placed side by side in the ice-box.

The Structure of the Fatty Acid Molecule.—Oleic acid is the most important of the unsaturated fatty acids, occurring as it does

[4] Jensen, O., Beiträge zur Kenntnis und Analyse der Flüchtigen Fettsäuren in Palmfetten und Butter, *Z. Nahr. Genussm.*, 10 : 265–283 (1905).

rather generally throughout the vegetable and animal kingdoms. The structure of oleic acid was established by the following steps:

1. An elementary analysis showed only carbon, hydrogen, and oxygen to be present in a proportion corresponding to the empirical formula $(C_9H_{17}O)_x$.

2. A molecular weight determination showed that the formula must be $C_{18}H_{34}O_2$.

3. Esterification and titration with bases indicated the presence of a single carboxyl group.

4. The addition of iodine indicated the presence of one double bond, forming a di-iodide (bromine could not be used, inasmuch as it substituted in the compound).

5. Stearic acid was obtained upon the reduction of oleic acid (the addition of two hydrogen atoms).

6. When oleic acid undergoes oxidation, a dihydroxy stearic acid is first formed, the molecule then breaking at the double bond upon further oxidation into a saturated C_9 monobasic acid and a saturated C_9 dibasic acid. Both of these C_9 acids were found to be straight-chain carbon compounds. Therefore, the double bond of oleic acid was in the center of the carbon chain, and the formula could be written

$$CH_3(CH_2)_7—CH{=}CH(CH_2)_7—COOH.$$

This formula, however, does not represent the true formula, for such a compound can exist in two stereomeric forms,

$$\begin{array}{l} CH_3—(CH_2)_7—CH \\ \qquad\qquad \| \\ HOOC—(CH_2)_7—CH \end{array}$$

(*Cis* form)

and

$$\begin{array}{l} CH_3—(CH_2)_7—CH \\ \qquad\qquad \| \\ \qquad\qquad CH—(CH_2)_7—COOH \end{array}$$

(*Trans* form)

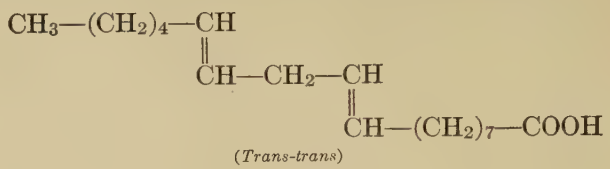
One of these is oleic acid (M.P. 14°); the other is eläidic acid (M.P. 45°). As already noted, eläidic acid does not occur in nature. Oleic acid is transformed into eläidic acid in the presence of a trace of NO_2.

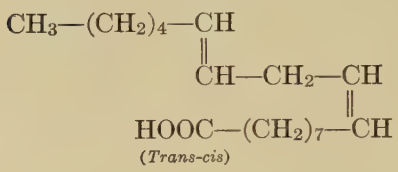
The formation of stearic acid by the hydrogenation of oleic acid proves the formula of stearic acid.

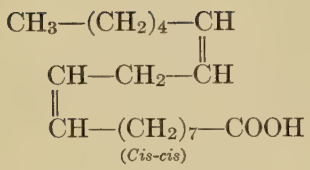
It is relatively easy to determine the structure of the molecule of a fatty acid which contains only a single double bond. However, the determination of the structure of those fatty acids containing two or more double bonds is much more difficult. Linoleic acid *probably* has the formula,

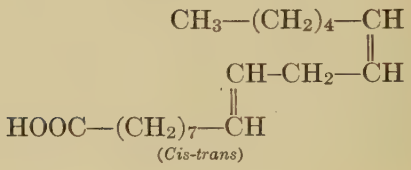
$$CH_3—(CH_2)_4—CH{=}CH—CH_2—CH{=}CH—(CH_2)_7—COOH.$$

Such a formula has four possible *cis-trans* stereoisomers (cf. Nicolet and Cox[5]).









Similarly, elomargaric acid,

$$CH_3—(CH_2)_3—CH=CH—CH=CH—(CH_2)_9—COOH,$$

has four possible stereomeric forms corresponding to those noted for linoleic acid. Elomargaric acid occurs in Chinese wood oil, from 90 to 95 per cent of the glycerides of Chinese wood oil being present as the esters of this acid (cf. Rhodes and Welz[6]).

The problem of *cis-trans* isomerism has taken on added significance following the study of Cooper and Edgar[7] as to the comparative biological effects of *cis-trans* isomers. These workers studied maleic, fumaric, dibromosuccinic, succinic, citraconic, mesaconic, and itaconic acids. They found that the *trans*-acids were superior to their *cis*-isomerides in regard to (*a*) bactericidal action, (*b*) as protein precipitants, and (*c*) as " activating " enzymatic reactions. The *cis*-acids, they found to be

[5] Nicolet, B. H., and Cox, H. L., C_{18} Acids. III. Four Tetrahydroxy-Stearic Acids Derived from Linolic Acid, and Their Significance with Regard to the Linolic Acid of Common Oils, *J. Am. Chem. Soc.*, 44 : 144–152 (1922).

[6] Rhodes, F. H., and Welz, C. J., The Chemistry of Chinese Wood Oil, *Ind. Eng. Chem.*, 19 : 68–73 (1927).

[7] Cooper, E. A., and Edgar, S. H., The Biological Significance of *Cis-Trans* Isomerism, *Biochem. J.*, 20 : 1060–1070 (1926).

more strongly adsorbed by proteins than were the *trans*-acids. They note that fumaric acid is a stronger disinfectant than is phenol and is much less toxic to higher animals, and suggest that *trans*-derivatives may prove to be useful germicides.

THE HYDROGENATION OF OILS.—The pioneer work in this field was due to the activities of Sabatier who discovered that certain metals catalyzed the reaction, $H_2 + R—CH{=}CH—R \rightarrow R—CH_2—CH_2—R$. The principal metals which have been employed are iron, cobalt, copper, nickel, platinum, and palladium. The reaction is carried out at an elevated temperature in the presence of a hydrogen atmosphere and the catalyst (cf. Sabatier[8]).

By the means of catalytic hydrogenation, unsaturated hydrocarbons, such as ethylene, acetylene, etc., may be hydrogenated to the C_nH_{2n+2} series. Benzene is reduced to cyclohexane (hexahydrobenzene). The monosaccharides are readily reduced to the corresponding alcohols, etc.

An excellent history of the commercial application of the process of nydrogenation to oils is presented in the court decision of the case of Proctor and Gamble Company versus Berlin Mills Company[9] (cf. also Richardson[10, 11]).

The lard substitutes, such as "Crisco," "Snowdrift," etc., do not represent anywhere nearly a complete hydrogenation of the vegetable oils. If the vegetable oils were completely hydrogenated, the resulting product would be brittle and similar to stearin or tallow. "Crisco" contains 20 to 25 per cent of saturated fats, 65 to 75 per cent of oleins, and 5 to 10 per cent of linoleins.

The opposite of hydrogenation, *i.e.*, dehydrogenation, in which hydrogen is abstracted from the oils, has been more or less successfully accomplished on a small scale by heating the oils with catalysts. Dehydrogenation does not appear to have been practiced on a large commercial scale comparable to the hydrogenation industry. At least there is no general knowledge of such large-scale operation. If dehydrogenation on a large scale could be readily accomplished, the *drying oils* for paints could be readily made from the natural non-drying oils.

[8] Sabatier, P., How I Have Been Led to the Direct Hydrogenation Method by Metallic Catalysts, *Ind. Eng. Chem.*, 18 : 1005–1008 (1926).

[9] Hydrogenation of Oils, *J. Ind. Eng. Chem.*, 9 : 1146–1148 (1917).

[10] Richardson, A. S., Knuth, C. A., and Milligan, C. H., Heterogeneous Catalysis, I. Selective Action of Catalytic Nickel in Hydrogenation of Certain Vegetable Oils. II. Hydrogenation of Marine Oils, *Ind. Eng. Chem.*, 16 : 519–522 (1924); 17 : 80–83 (1925).

[11] Richardson, A. S., and Snoddy, A. O., Heterogeneous Catalysis. III. Hydrogenation of Cottonseed Oil with Platinum, *Ind. Eng. Chem.*, 18 : 570–571 (1926).

Ozonides.—Many organic compounds containing unsaturated linkages add ozone at the double bond. Thus, ethylene adds one molecule of ozone to form an ozonide,

$$CH_2{=}CH_2 + O_3 = \begin{matrix} CH_2 & - & - & CH_2 \\ | & & & | \\ O & - & O & - & O \end{matrix}.$$

When an ozonide is treated with water, it breaks up into aldehydes, ketones, or peroxides of aldehydes or ketones, and from a study of the resulting decomposition products, we can decide at what position the unsaturated double bond occurred in the original organic compound. Thus, oleic acid forms an ozonide in acetic acid solution, and on treatment of the oleic acid ozonide with water, it decomposes into hydrogen peroxide, pelargonic acid, and azelaic acid semi-aldehyde,

$$\begin{matrix} C_8H_{17} & - & CH & - & - & CH & - & (CH_2)_7 & - & COOH \\ & & | & & & | & & & & \\ & & O & - & O & - & O & & & \end{matrix} + H_2O$$

$$= H_2O_2 + C_8H_{17}{-}CHO + CHO{-}(CH_2)_7{-}COOH.$$

$$C_8H_{17}CHO + H_2O_2 = C_8H_{17}COOH + H_2O.$$

This confirms the original structure proposed for oleic acid, *i.e.*, that the double bond is in the center of the molecule.

Spontaneous Changes in Fats and Oils.—Rancidity.—The term, rancidity, is used in two different industries to represent two entirely different changes which take place in fats and oils, (1) the hydrolysis of the glycerides, with the liberation of free fatty acids, and (2) the oxidation of fats and oils containing unsaturated acids, resulting in the formation of aldehydes, ketones, and acids, having a lower molecular weight than the acids which were naturally present. The term, rancidity, as employed in the oil and fat industry usually applies to the oxidative process, whereas in the dairy industry it applies to hydrolysis with the corresponding liberation of butyric acid which can be detected by its characteristic odor. The workers in the dairy industry refer to the oxidative process as producing " tallowy butter " in contrast to the " rancid butter " produced by the hydrolytic process. As a general rule, both oxidation and hydrolysis occur simultaneously, so that probably from the industrial standpoint no sharp line of demarcation can be drawn, although from the chemical standpoint the two mechanisms are sharply differentiated.

In the process of hydrolysis there is always an increase in titratable acidity. The presence of any trace of lipase (the fat-splitting enzyme) will hasten and promote this type of rancidity.

Oxygen is necessary in order to produce the oxidation type of rancidity. Heat, light, and moisture, together with the presence of certain metals which catalyze the reaction, hasten the oxidative process. Holm notes that the greatest effect of light occurs when the light has a wave length of approximately 3600 Å. The character of the fat and the enviromental conditions determine whether one obtains aldehydes, acids, ketones, etc., so that an increased titration value need not occur, but usually does occur in oxidative rancidity. The Kreis color test (HCl + phloroglucinol + ether + the suspected fat) is the usual test which is employed to detect deterioration due to oxidative rancidity, although a positive Kreis test in the crude oil may be due, not to decomposition products of the fat, but to substances derived from the seed from which the fat or oil was obtained (cf. Smith[12]).

One feature of oxidative rancidity which contrasts this process snarply with the hydrolytic process is that the glycerol in oxidative rancidity disappears practically as fast as it is formed, being probably oxidized immediately on formation.

The strong odor characteristic of the lower fatty acids, especially of butyric acid, is readily produced in milk fat upon slight hydrolysis. Most other fats contain relatively small amounts of the lower fatty acids and much larger amounts of the higher fatty acids, stearic, palmitic, etc., which are almost odorless. Accordingly, hydrolysis of such fats produces comparatively little off-odor. The tallowy odor is produced by the oxidation of the unsaturated fatty acids and the formation of aldehydes and ketones. The oxidation of oleic acid is mainly responsible for the intense tallowy odor. Oxidation of linoleic acid produces less off-odors, while the oxidation of linolenic acid produces very slight amounts of off-odors. Milk fat accordingly contains the particular composition of fatty acids which makes possible an intense odor, resulting from either the hydrolytic or oxidative type of rancidity (cf. Holm and Greenbank[13-15]).

Holm and his coworkers,[16, 17] as well as others,[18] have shown that

[12] Smith, W. B., The Kreis Reaction of Cottonseed-Oil Products, *J. Ind. Eng. Chem.*, 12 : 764–766 (1920).

[13] Holm, G. E., and Greenbank, G. R., Quantitative Aspects of the Kreis Test, *Ind. Eng. Chem.*, 15 : 1051–1053 (1923).

[14] Holm, G. E., and Greenbank, G. R., Quantitative Aspects of the Kreis Test—II. *Ind. Eng. Chem.*, 16 : 518 (1924).

[15] Greenbank, G. R., and Holm, G. E., Some Factors Concerned in the Autoxidation of Fats, *Ind. Eng. Chem.*, 16 : 598–601 (1924).

[16] Greenbank, G. R., and Holm, G. E., Measurement of Susceptibility of Fats to Oxidation, *Ind. Eng. Chem.*, 17 : 625 (1925).

[17] Holm, G. E., and Greenbank, G. R., The Keeping Quality of Butterfat, with

the oxidative process can be divided into two periods, (1) the period of induction, and (2) the period of active oxygen absorption.

During the period of induction there is a negligible absorption of oxygen, and the susceptibility of a fat to oxidation may be determined by ascertaining under specified conditions the relative length of the induction period. Figure 126, taken from the data of Triebold, shows the variation of the induction period, in terms of rate of oxygen absorption plotted against time, for several samples of lard. There is a great variation in regard to the length of the induction period between the various samples, and there appears to be a marked correlation between the ease with which these various samples of fats became rancid when used in baked products and the shortness of the induction period. The form of the curves for oxygen absorption strongly suggests an autocatalytic reaction.

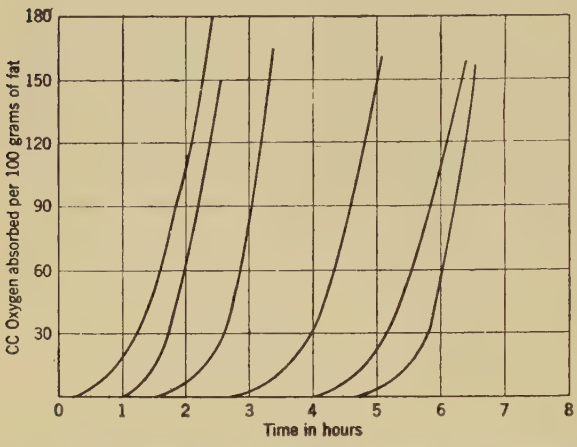


FIG. 126.—Showing the variation in the length of induction period of oxygen absorption for six commercial samples of lard. Temp. 95° C. (Data of Triebold.)

The presence of free acids greatly increases the susceptibility of fat to autoxidation. On the other hand, the addition of compounds containing hydroxyl groups or the presence of water decreases susceptibility to autoxidation. Holm, Greenbank, and Deysher[19] note that substances containing hydroxyl groups delay the onset of active oxygen absorption or lengthen the induction period but do not seem to materially alter the rate of absorption once it has begun. They note that a hydroxyl group attached to the unsaturated carbon chain of a fatty acid (ricinoleic acid) alters the rate of

Special Reference to Milk Powder, *Proc. World's Dairy Congress*, 2 : 1253–1265 (1923).

[18] Triebold, H. O., The Induction Period of Oxygen Absorption of Edible Fats, Master's thesis filed in the Library, University of Minnesota (1926).

[19] Holm, G. E., Greenbank, G. R., and Deysher, E. F., Susceptibility of Fats to Autoxidation, *Ind. Eng. Chem.*, 19 : 156–158 (1927).

oxygen absorption. Certain of their data are given in Figs. 127 and 128.

The period of active oxygen absorption is characterized by the extremely rapid rate of absorption by those acids, such as linoleic and linolenic which are characteristic of the drying oils. The rate of oxygen absorption by oleic acid is decidedly slower, and, as already noted, ricinoleic acid is characterized by a very slow rate of oxygen absorption.

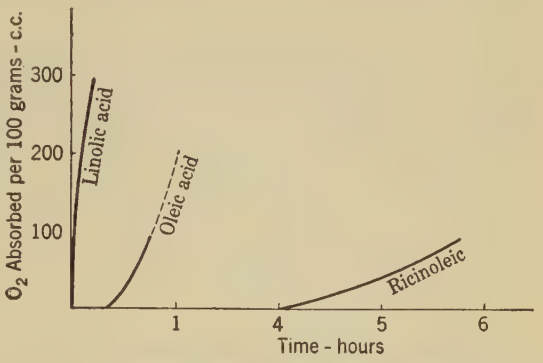


FIG. 127.—Showing the rate of oxygen absorption and the length of induction period characteristic of certain unsaturated fatty acids. (Data of Holm.)

Holm suggests that the first reaction is the formation of "moloxides"[20] or loosely combined compounds which in themselves possess potential oxidizing ability. This stage is followed by the oxidation itself.

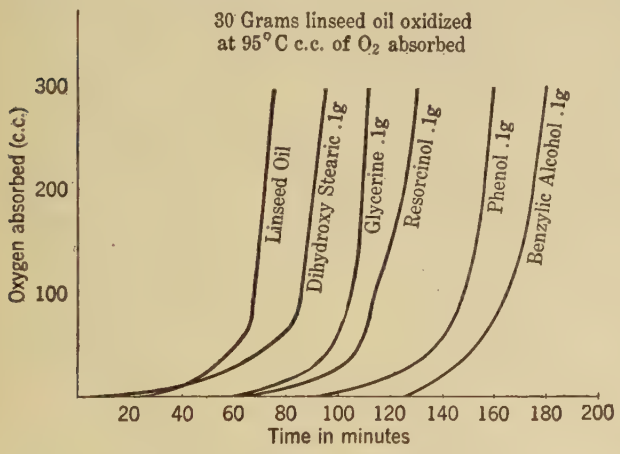


FIG. 128.—Showing the effect of anticatalysts upon the autoxidation of linseed oil. (Data of Holm et al.)

The reaction which is involved is presumably identical with that involved in the formation of the ozonide, excepting that the first stage appears to be the addition of molecular oxygen instead of ozone. If water is present a partial decomposition of the peroxide occurs, with the formation of an oxide and hydrogen peroxide. Additional oxide then combines with the oxygen from hydrogen peroxide to form an ozonide which decomposes into aldehydes and aldehyde acids, as already noted. Holm

[20] Staudinger, H., Über Autoxydation organischer Verbindungen. III. Über Autoxydation des asymn. Diphenyl-äthylens, *Ber.*, 58B : 1075–1079 (1925).

suggests that the above explanation for the mechanism would have to be altered in the event that no water is present. In such a case, it would be difficult to base an explanation on the hydrogen peroxide theory, and one might have to assume that "active" oxygen takes part in the reaction.

Drying Oils.—These are the oils which are valuable to the paint and varnish industry and include especially those oils which contain glycerides in which the acids, at least in part, belong to the linoleic and linolenic series. The drying oils include such oils as linseed oil, poppy-seed oil, sunflower-seed oil, hempseed oil, walnut oil, etc., and are characterized by the fact that the oils readily absorb oxygen from the air, and thicken and resinify. If they are exposed in a thin layer, such oils dry to a tough, elastic film.

Certain "semi-drying" oils are intermediate in drying properties between the true drying oils and the non-drying oils. These semi-drying oils include cottonseed oil, soybean oil, corn oil, rapeseed oil, the oils from melon seeds, pumpkin oil, mustard oil, etc.

The non-drying oils are such oils as castor oil, olive oil, peanut oil, etc.

The iodine number of an oil is an indication as to whether or not the oil is a drying oil, a semi-drying oil, or a non-drying oil. The higher the iodine number, the greater is its drying property, although the actual test for a drying oil is the exposure to air of a film of the oil on a glass plate. A drying oil should form a dry film in from three to six days; a semi-drying oil will be somewhat sticky even after a week of exposure, whereas a non-drying oil would be soft and practically unaltered after eighteen or twenty days of exposure to the air.

Saponification of Fats. The Formation of "Soaps."—Saponification of fats by alkalies brings about hydrolysis, with the formation of glycerol and the salts of the fatty acids. The salts of the higher fatty acids are soaps. The colloidal behavior of soaps as colloidal systems in themselves and as emulsifying agents has already been noted (cf. also Martin Fischer,[21] McBain,[22-24] and Nuttall[25]).

[21] Fischer, Martin H., with the collaboration of McLaughlin, G. D., and Hooker, M. O., Soaps and Proteins, John Wiley and Sons, Inc., New York (1921).

[22] McBain, J. W., Colloidal Chemistry of Soap, Pt. I. Solutions, Third Report on Colloid Chemistry, Brit. Assoc. Advancement Sci., pp. 2–31, London (1920).

[23] McBain, J. W., and Walls, E., Colloid Chemistry of Soap, Pt. II. The Soap Boiling Processes, Fourth Report on Colloid Chemistry, Brit. Assoc. Advancement of Sci., pp. 244–263, London (1922).

[24] McBain, J. W., The States of Matter Exemplified by a Typical Colloid. Soap and the Soap Boiling Processes, Chap. V. of Colloid Chemistry, Theoretical and

Six general methods have been used to saponify fats:

1. The acidification process, in which the fat is heated with dilute sulfuric acid or hydrochloric acid under pressure at a temperature exceeding 100° C.

2. The autoclave process, in which the fats are treated with superheated steam in the presence of a small amount of calcium hydroxide.

3. Saponification with sodium hydroxide or potassium hydroxide solutions at the boiling temperature.

4. The cold saponification process, in which the required amount of concentrated alkali is added and the mixture is allowed to stand for several days.

5. The Twitchell process,[26] in which saponification is carried out in the presence of sulfobenzenestearic acid which acts as a catalyst.

6. The biological method, in which lipase or lipolytic enzymes are added to the fat, resulting in the formation of the free fatty acids and glycerol.

Glycerol, the Alcohol Present in Fats.—Glycerol is a triatomic alcohol, having the formula, $CH_2OH—CHOH—CH_2OH$. Until recently the commercial source of glycerol has been the waste water of soap factories. Within the last few years, stimulated by the necessity of finding new sources of glycerol for the preparation of nitroglycerine, attention has been directed to the biological synthesis of this important substance. It has been prepared by bacterial and yeast fermentations of carbohydrates[27] (cf., also Barger,[28] Guilliermond,[29] and Connstein and Lüdecke[30]).

In the case of yeast fermentation, certain strains of yeast were found to be resistant to sodium sulfite. When they were added to solutions containing dextrose and sodium sulfite, alcoholic fermentation was largely inhibited, whereas glycerol fermentation was stimulated.

Glycerol is miscible in water in all proportions, and when strongly

Applied, Vol. I, edited by Jerome Alexander, Chemical Catalog Company, Inc., New York (1926).

[25] Nuttall, W. H., Industrial Application of Wetting Power, Fifth Report on Colloid Chemistry, Brit. Assoc. Advancement Sci., pp. 38–47 (1923).

[26] Perkin Medal Award to Ernst Twitchell, *J. Ind. Eng. Chem.*, 9 : 192–198 (1917).

[27] Anon., Chemistry of the Fermentation Process of Glycerine Production, *Chem. Age*, 28 : 352–354 (1920).

[28] Barger, G., Enzymes and Fermentation, *Annual Reports, Chem. Soc.*, London, 16 : 166–170 (1919).

[29] Guilliermond, A., The Yeasts, (translated and revised by Tanner, F. W.), John Wiley and Sons, Inc., New York (1920).

[30] Connstein, W., and Lüdecke, K., Über Glycerin-Gewinnung durch Gärung, *Ber.*, 52B : 1385–1391 (1919).

heated either alone or in the presence of a dehydrating agent, such as potassium bisulfate, it decomposes to form acrolein, $CH_2{=}CH{-}CHO$. Acrolein possesses a very penetrating, acrid odor. The formation of acrolein may be regarded as a test for the presence of glycerol. The odor of acrolein is noticeable when a tallow candle is blown out or when a fat is heated to too high a temperature.

The oxidation products of glycerol depend upon the oxidizing agent which is employed, *i.e.*, the oxidation potential. It may be oxidized to acrolein, to glyceric aldehyde, or completely oxidized to carbon dioxide and water. As one might expect, inasmuch as it is closely related to sugars, glycerol is relatively easily oxidized. Glycerol may be regarded as the alcohol which is formed by the reduction of the triose, glycerose. It shows the sugar character, inasmuch as it reduces many metallic salts, forms glycerolates with alkalies, is esterfied by acids, *e.g.*, glycerol phosphoric acid ester (*vide infra*), and in fact has all the properties which might be expected to accompany a trihydroxy alcohol.

The Waxes.—Waxes differ from fats in that they are esters of mono- (or in some instances di-) hydroxy saturated alcohols or of sterols with certain of the higher fatty acids. Certain of the *monohydroxy alcohols* of the $C_nH_{2n+2}O$ series which occur in waxes are:

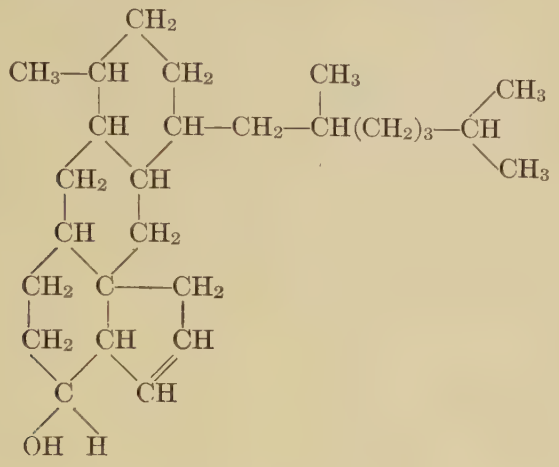
Cetyl alcohol.....	$C_{16}H_{33}OH$	occurs in spermaceti as the palmitate
Octodecyl alcohol.....	$C_{18}H_{37}OH$	occurs in spermaceti as the palmitate.
Eicosyl alcohol.....	$C_{20}H_{41}OH$	
Carnaubyl alcohol.....	$C_{24}H_{49}OH$	occurs in wool fat.
Ceryl alcohol.....	$C_{26}H_{53}OH$	occurs in Chinese wax, as cerotate (C_{26}), in poppy wax as palmitate, and in insect wax.
Myricyl or melissyl alcohol	$C_{30}H_{61}OH$	occurs in carnauba wax as cerotate, and in beeswax as palmitate.

Cyclic alcohols—sterols (Greek, στερεος = solid, and "ol" ending, meaning alcohol).

Most of these monohydroxy alcohols occur in nature only in the waxes.

A much larger fraction of the waxes appears in the so-called "unsaponifiable matter," than is the case in the fats and oils. In the waxes the unsaponifiable matter consists largely of the alcohols of high melting point which were esterified by the fatty acids. Thus, the unsaponifiable matter of liquid waxes may range from 31 to 43 per cent, and of the solid waxes from 43 to 55 per cent. Hydrocarbons may also occur as constituents of solid waxes. Sterols, as a rule, are practically absent. Waxes are much more difficultly saponified than are the fats and oils which are esters of glycerol, and usually require saponification by alcoholic potassium hydroxide.

THE STEROLS.—**A. The Animal Sterols.**—Cholesterol is the chief animal sterol and apparently occurs as a constituent of all animal cells, although it is much more abundant in nervous tissue than in other animal tissues. Cholesterol is a cyclic monatomic alcohol containing one double bond, and according to Windaus[31] probably possesses the formula,



and having the empirical formula, $C_{27}H_{45}OH$.

Cholesterol can be conveniently prepared from sheep or hog brains, which after maceration are mixed with anhydrous calcium sulfate (plaster of Paris) in sufficient quantity so that when the plaster of Paris has been converted to gypsum, the water present in the biological material will have been bound as water of crystallization. The mass of brain tissue containing the gypsum is then powdered and extracted with ether. Crude cholesterol can be crystallized directly by concentrating the ether extract or by preparing the nonsaponifiable residue from such an ether extract and fractionally crystallizing the nonsaponifiable residue.

Cholesterol yields three color reactions, (1) the Liebermann-Burchard reaction, a violet color produced by cholesterol treated with chloroform, acetic anhydride, and sulfuric acid, (2) the Salkowski reaction, a cherry-red coloration produced by cholesterol in the presence of sulfuric acid and chloroform, and (3) the reaction suggested by Steinle and Kahlenberg.[32] These workers have described a new color reaction by

[31] Windaus, A., Abbau- und Aufbauversuche im Gebiete der Sterine, *Abderhalden's Handb. biol. Arbeitsmeth.*, Abt. I, Teil 6, Heft 1 : 169–208 (1922).

[32] Steinle, J. V., and Kahlenberg, L., A New Method for the Identification and Estimation of Cholesterol and Certain Other Compounds, *J. Biol. Chem.*, 67 : 425–467 (1926).

treating cholesterol in chloroform solution with antimony pentachloride. They state that a simple addition product is formed which, dissolved in the chloroform, yields a clear purple solution changing to a cobalt-blue on exposure to light. They also state that the color reaction is quantitative and can be used for the estimation of cholesterol by colorimetric methods. They note that the reaction is also quantitative for phytosterol but that the color does not develop as readily as it does with cholesterol.

Cholesterol may be gravimetrically estimated as the cholesteride of digitonin, inasmuch as cholesterol forms an insoluble precipitate with digitonin as has already been noted (cf. also Girardin and Spach[33]). It may be estimated by using Bloor's colorimetric method[34] (cf. also Myers and Wardell[35]), which is simply the adaptation of the Liebermann-Burchard reaction to quantitative technic. Cholesterol inhibits the action of saponins and prevents the hemolysis of red cells in the presence of saponins (cf. Ransom,[36] Hausmann,[37] and Abderhalden and Le Count[38]).

The esters of cholesterol do not inhibit the action of saponins. It would thus appear that cholesterol forms a definite compound with all saponins, as well as with digitonin. The presence of cholesterol likewise inhibits or at least retards the action of lipolytic enzymes. Cholesterol esterified with oleic acid and palmitic acid occurs in small amounts in the blood serum.

Several derivatives or isomers of cholesterol have been isolated from animal sources. Thus, *coprosterol* occurs in human feces and undoubtedly arises from the alteration of the original cholesterol molecule. Windaus notes that coprosterol is apparently identical with cholesterol but lacks the unsaturation represented by the double bond. He has been able to prepare coprosterol by the catalytic hydrogenation of cholesterol, using nickel in a hydrogen atmosphere at 200° C.

[33] Girardin, R., and Spach, E., Sur le microdosage pondéral du cholestérol, *Bull. soc. chim. biol.*, 8 : 813–815 (1926).

[34] Bloor, W. R., The Determination of Cholesterol in Blood, *J. Biol. Chem.*, 24 : 227–231 (1916).

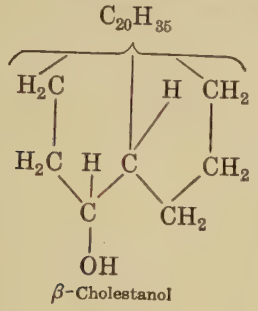
[35] Myers, V. C., and Wardell, E. L., The Colorimetric Estimation of Cholesterol in Blood, with a Note on the Estimation of Coprosterol in Feces, *J. Biol. Chem.*, 36 : 147–156 (1918).

[36] Ransom, F., Saponin und sein Gegengift, *Deut. med. Wochschr.*, 27 : 194–196 (1901).

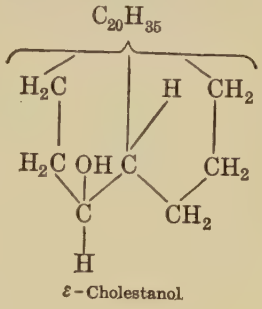
[37] Hausmann, W., Über die Engiftung des Saponins durch Cholesterin, *Beitr. chem. Physiol.*, 6 : 567–580 (1905).

[38] Abderhalden, E., and Le Count, E. R., Die Beziehungen zwischen Cholesterin, Lecithin und Cobragift, Tetanustoxin, Saponin und Solanum, *Z. exp. Path. Therap.*, 2 : 199–215 (1906).

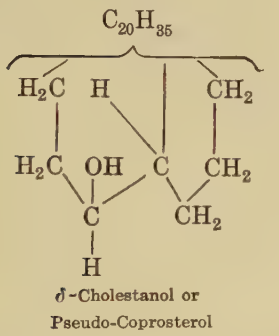
Inasmuch as cholesterol contains several asymmetric carbon atoms, the existence of a number of isomeric cholesterols, possessing the same empirical formula and the same graphic formula except for space relationships, is possible. Windaus notes that by the reduction of cholesterol a mixture of isomeric compounds is formed, to one of which he has tentatively assigned the space relationships characteristic of coprosterol. He gives the following formulae for the four isomerides.



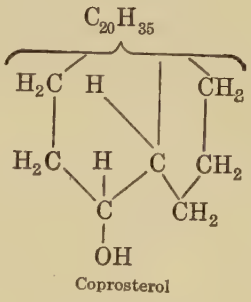
β-Cholestanol



ε-Cholestanol



δ-Cholestanol or
Pseudo-Coprosterol



Coprosterol

Windaus notes that the separation of these four isomers presents great difficulties. However, β-cholestanol and coprosterol are precipitated by digitonin, whereas ε-cholestanol and δ-cholestanol are not precipitated by digitonin. By the use of other reagents and transformations, he was able to make eventually a separation of the four compounds.

An isomer of cholesterol, *i.e.*, isocholesterol, occurs, together with cholesterol in wool fat. It is distinguished from cholesterol by having somewhat different color reactions and possessing an optical rotation of $[\alpha]_D$ $+60°$, in contrast to the optical rotation of cholesterol, $[\alpha]_D$ $-31.59°$.

B. The Vegetable Sterols.—A number of sterols have been isolated from various vegetable sources. Bömer[39] notes that the plant sterols

[39] Bömer, A., Allgemeine Methoden der Darstellung und Untersuchung der Fette, *Abderhalden's Handb. biol. Arbeitsmeth.*, Abt. I, Teil 6, Heft 2 : 301–568 (1925).

may be grouped under the general head of phytosterols, and that in this group *sitosterol*, $C_{27}H_{44}O + H_2O$, *brassicasterol*, $C_{28}H_{46}O + H_2O$, and *stigmasterol*, $C_{30}H_{48}O + H_2O$ or $C_{30}H_{50}O + H_2O$, have been identified by Windaus as individual compounds. Bömer notes that sitosterol occurs in wheat oil, cottonseed oil, and linseed oil. Both sitosterol and stigmasterol occur in cocoa butter, the fat of the calabar bean, and in cocoanut oil, and both sitosterol and brassicasterol occur in rapeseed oil. To the above sterols we must of necessity add *ergosterol*, first isolated by Tanret from ergot. Undoubtedly, there are many sterols of plant origin, differing slightly in empirical formula and in the position and the number of double bonds which are present in the molecule, all of which are more or less closely related to cholesterol insofar as their molecular configuration is concerned.

Within the last few years we have seen a revival of interest in the study of the sterols both from the standpoint of their isolation and identification and from the standpoint of their physical and chemical properties and physiological behavior. Some workers have attempted to distinguish two groups of sterols in plants, assigning the phytosterols to the higher plants and the ergosterols to the plants of lower orders. Recent work, however, which will be noted shortly, indicates that ergosterol may occur, at least in small amounts, in many of the higher plants.

In the plants the sterols may occur either combined in the form of glucosides[40] or as esters of the higher fatty acids, and some workers claim that they may occur as the free sterol. Ellis,[41] in a study of the phytosterols of wheat plants, notes that the compound isolated from wheat bran differs from the compound which is present in the endosperm and that a third compound, $C_{29}H_{42}O$, is present in green shoots.

Anderson[42] isolated two different sterols from the endosperm of corn, one of which appeared to be identical with sitosterol, the other a dihydrositosterol, $C_{27}H_{47}OH$, a saturated sterol which did not show the Liebermann-Burchard reaction. In later studies of the phytosterol of wheat, Anderson and Nabenhauer[43] again isolated a sitosterol and found a dihydrositosterol, having the same formula as the compound isolated from corn endosperm. The dihydrositosterol occurred in somewhat

[40] Power, F. B., and Chestnut, V. K., Non-Volatile Constituents of the Cotton Plant, *J. Am. Chem. Soc.*, 48 : 2721–2737 (1926).

[41] Ellis, M. T., Contributions to Our Knowledge of the Plant Sterols. Pt. I. The Sterol Content of Wheat (*Triticum sativum*), *Biochem. J.*, 12 : 160–172 (1918).

[42] Anderson, R. J., The Phytosterols of the Endosperm of Corn, *J. Am. Chem. Soc.*, 46 : 1450–1460 (1924).

[43] Anderson, R. J., and Nabenhauer, F. P., The Phytosterols of Wheat Endosperm, *J. Am. Chem. Soc.*, 46 : 1717–1721 (1924).

larger quantities in wheat bran than it did in the wheat endosperm. Anderson and Shriner,[44] in a study of corn oil isolated a small quantity of stigmasterol, $C_{30}H_{50}O$, dihydrositosterol, $C_{27}H_{47}OH$, and a mixture containing at least three isomeric sterols identical in composition with sitosterol, having the formula, $C_{27}H_{45}OH$, but differing from each other in certain physical properties. These isomers, Anderson and Shriner designated as α-, β-, and γ-sitosterol. Anderson, Shriner, and Burr,[45] in a similar study of wheat oil, again found a mixture of α-, β-, and γ-sitosterol.

Marston[46] has isolated a mycosterol from the fungus, *Boletus granulatus*, having the formula, $C_{30}H_{48}O_2$, possessing one hydroxyl group but having no double bond. Apparently the same sterol has been isolated by Ikeguchi[47] from *Collybia shiitake*, *Armillaria edodes*, and *Hydnum asparatum*. Ikeguchi, from his study of this sterol, concludes that it is a saturated sterol, containing no double bonds and possessing two hydroxyl groups, accordingly having the formula, $C_{30}H_{46}(OH)_2$.

A great stimulus to research in the field of the sterols followed the discovery of Rosenheim and Webster,[48] Steenbock and Black,[49] and Hess, Weinstock, and Helman,[50] who noted independently and at approximately the same time that apparently pure samples of cholesterol, when irradiated with ultraviolet light acquired the properties of replacing the antirachitic vitamin, vitamin D, in the diet of an animal. Following these discoveries many workers irradiated oils and fats from various plant and animal sources, irradiated various foodstuffs and various sterols, and found rather generally that the sterol fraction acquired antirachitic properties following irradiation. As the study progressed, however, it was noted that all samples of sterols did not acquire the antirachitic property to the same degree, and a number of workers found almost simultaneously that cholesterol which had been purified

[44] Anderson, R. J., and Shriner, R. L., The Phytosterols of Corn Oil, *J. Am. Chem. Soc.*, 48 : 2976–2986 (1926).

[45] Anderson, R. J., Shriner, R. L., and Burr, G. O., The Phytosterols of Wheat Germ Oil, *J. Am. Chem. Soc.*, 48 : 2987–2996 (1926).

[46] Marston, H. R., The Sterol of *Boletus granulatus*, *Australian J. Exp. Biol. Med. Sci.*, 1 : 53–55 (1924).

[47] Ikeguchi, T., A New Sterol, *J. Biol. Chem.*, 40 : 175–182 (1919).

[48] Rosenheim, O., and Webster, T. A., Rickets and Cholesterol, *Lancet*, 208: 1025–1026 (1925).

[49] Steenbock, H., and Black, A., Fat-Soluble Vitamins. XXIII. The Induction of Growth-Promoting and Calcifying Properties in Fats and Their Unsaponifiable Constituents by Exposure to Light, *J. Biol. Chem.*, 64 : 263–298 (1925).

[50] Hess, A. F., Weinstock, M., and Helman, F. D., The Antirachitic Value of Irradiated Phytosterol and Cholesterol. I., *J. Biol. Chem.*, 63 : 305–308 (1925).

by chemical methods had lost the property of becoming antirachitic when irradiated (cf. Rosenheim and Webster[51]).

Prior to this time, Hess, Weinstock, and Sherman[52] had noted that irradiated cholesterol lost its antirachitic properties when it was recrystallized, and that the apparently pure cholesterol, which could be obtained from the recrystallization of irradiated cholesterol, could not again be activated by irradiation. They also noted[53] that following irradiation all of the sterol could not be precipitated by digitonin. At about the same time Rosenheim and Webster[54] observed that only the sterols obtained directly from plant or animal tissues, such as cholesterol, sitosterol, and ergosterol, could be activated by irradiation. The group of "excretory" sterols, including coprosterol, were not activated by irradiation, indicating that the presence of an unsaturated linkage was necessary in order that the substance should acquire antirachitic properties.

Hess and Anderson,[55] in a study of Anderson's α-, β-, and γ-sitosterols, found that the α-sitosterol became strongly antirachitic following irradiation, whereas the β- and γ-sitosterols acquired no antirachitic properties. At the time their paper was published the suggestion had been made that ergosterol was the precursor of vitamin D. They note that their β- and γ-sitosterols had been purified by chemical process (bromination) and suggest that possibly this process had destroyed any precursor of the antirachitic factor which may originally have been present in these fractions as an impurity. They state, "If ergosterol is the sole antirachitic precursor, it is evident that this sterol must be universally present in all fats of animal and of plant origin that are capable of activation by ultraviolet light. This is an exceedingly broad conception."

Rosenheim and Webster further noted that when cholesterol was purified by chemical means and had lost the property of becoming anti-

[51] Rosenheim, O., and Webster, T. A., Further Observations on the Photo-Chemical Formation of Vitamin D, *J. Soc. Chem. Ind.*, 45 : 932 (1926).

[52] Hess, A. F., Weinstock, M., and Sherman, E., The Antirachitic Value of Irradiated Cholesterol and Phytosterol. IV. Factors Influencing Its Biological Activity, *J. Biol. Chem.*, 66 : 145–160 (1925).

[53] Hess, A. F., Weinstock, M., and Sherman, E., The Antirachitic Value of Irradiated Cholesterol and Phytosterol. V. Chemical and Biological Changes, *J. Biol. Chem.*, 67 : 413–423 (1926).

[54] Rosenheim, O., and Webster, T. A., The Anti-Rachitic Properties of Irradiated Sterols, *Biochem. J.*, 20 : 537–544 (1926).

[55] Hess, A. F., and Anderson, R. J., The Antirachitic Value of Irradiated Cholesterol and Phytosterol. VIII. The Activation of Sterol Fractions by Ultra-Violet Irradiation, *J. Biol. Chem.*, 74 : 651–657 (1927).

rachitic by irradiation, it likewise no longer possessed a characteristic absorption spectrum band in the ultraviolet region,[56, 57] which is characteristic for cholesterol purified only by recrystallization. It appeared, therefore, as if some impurity were present in cholesterol purified by physical means and that the presence of this impurity accounted for the characteristic ultraviolet absorption band and for the acquiring of antirachitic properties by the cholesterol preparation.

Accordingly Rosenheim, Webster, Hess, and Windaus began an intensive investigation as to the nature of the impurity which might be present in the various sterol preparations and which might act as the provitamin of vitamin D. While these workers were attacking the problem in different laboratories, nevertheless through a friendly agreement, they were all kept informed of the progress of the work in their several institutions. Almost simultaneously Windaus and Hess,[58] and Rosenheim and Webster[59] announced the finding that neither cholesterol which contains one double bond, sitosterol containing one double bond, nor stigmasterol containing two double bonds acquired antirachitic properties on irradiation following their purification by chemical methods. They did observe, however, that ergosterol, which contains three double bonds and which Rosenheim and Webster[60] had previously found to be after irradiation "highly protective even in doses of 1 mg.," was apparently present in small amounts in all of the sterol preparations which could be activated by irradiation, and they announced that the provitamin of vitamin D is ergosterol.

Rosenheim and Webster[61] have presented a historical discussion of this announcement and note that if ergosterol is present in ordinary cholesterol to the extent of one part of ergosterol to 2000 parts of cholesterol, it will still be present in sufficient amount to account for the acquirement of antirachitic properties on irradiation by the supposedly pure samples of cholesterol. In this study Rosenheim and Webster

[56] Schlutz, F. W., and Ziegler, M. R., Some Spectroscopic Observations on Cod Liver Oil, *J. Biol. Chem.*, 69 : 415–419 (1926).

[57] Morton, R. A., Heilbron, I. M., and Kamm, E. D., The Absorption Spectrum of Ergosterol in Relation to the Photosynthetic Formation of Vitamin D, *J. Chem. Soc.*, London, 1927 : 2000–2005.

[58] Windaus, A., and Hess, A., Sterine und antirachitisches Vitamin, *Nachr. Ges. Wiss. Göttingen, Math. -physik. Klasse*, 1926 : 175–184.

[59] Rosenheim, O., and Webster, T. A., On the Nature of the Parent Substance of Vitamin D, *Lancet*, 212 : 306–307 (1927).

[60] Rosenheim, O., and Webster, T. A., The Anti-Rachitic Properties of Irradiated Sterols, *Biochem. J.*, 20 : 537–544 (1926).

[61] Rosenheim, O., and Webster, T. A., The Parent Substance of Vitamin D, *Biochem. J.*, 21 : 389–397 (1927).

state that a daily dosage of 1/10,000 of a milligram of irradiated ergosterol is sufficient to cure or prevent the occurrence of rickets in rats kept on a rachitic diet and that, while this amount was used in their experiments, they believe that as little as 1/50,000 of a milligram may be adequate. On this basis, one liter of highly potent cod-liver oil need not contain more than the equivalent of 5 milligrams of irradiated ergosterol.

As already indicated, ergosterol appears to be widely distributed in nature, and can be isolated from various sources including mushrooms, yeast, ergot, etc. A number of workers (cf. Reindel, Walter, and Rauch,[62] and Windaus and Brunken,[63] and Windaus and Linsert,[64] and Windaus and Borgeaud[65]) are actively engaged in the attempt to elucidate the change taking place in ergosterol upon irradiation. Windaus and Linsert note that the ergosterol is changed into a compound or mixture of compounds having high antirachitic activity and that accompanying this change there is a loss in the ability of ergosterol to form an insoluble precipitate with digitonin, a marked change in the optical activity, a change in the ultraviolet absorption spectra, and an increase in solubility.

Ergosterol can be rather readily obtained in crystalline form. Following irradiation by ultraviolet light, the antirachitic factor has not as yet been obtained as a crystalline product. Both the original ergosterol and the irradiated ergosterol possess the same molecular weight; both contain one free hydroxyl group, and catalytic reduction indicates that there has been no change in the number of unsaturated linkages in the molecule. The nature of the chemical change which has taken place must therefore involve some intramolecular rearrangement, as is evidenced by the change in physical properties, particularly the change in absorption spectra and optical rotation. Windaus suggests that vitamin D is an isomer of ergosterol, and that ergosterol is quantitatively transformed into vitamin D by the ultraviolet light.

The sterols are contained in the unsaponifiable matter of fats and oils, and are usually isolated by saponifying the fats, drying the soaps which are formed, and extracting these dry soaps with dry ether. The sterols are extracted by the ether, whereas the soaps are insoluble. The sterols are not water soluble, but in the saponified fat are dispersed as an

[62] Reindel, F., Walter, E., and Rauch, H., Über das Ergosterin der Hefe. I. *Ann.*, 452 : 34–46 (1927).

[63] Windaus, A., and Brunken, J., Über die photochemische Oxydation des Ergosterins, *Ann.*, 460 : 225–235 (1928).

[64] Windaus, A., and Linsert, O., Über die Ultraviolett-Bestrahlung des Dehydroergosterins, *Ann.*, 465 : 148–166 (1928).

[65] Windaus, A., and Borgeaud, P., Über die photochemische Dehydrierung des Ergosterins, *Ann.*, 460 : 235–239 (1928).

emulsion in the presence of the soap solution. Cottonseed oil and corn oil yield about one per cent of unsaponifiable matter. Olive oil yields somewhat less than one per cent, and wheat oil about 2.5 per cent (cf. Ball[66]). Fish and liver oils may contain as high as 10 to 20 per cent of unsaponifiable matter. In such instances, however, other substances, largely hydrocarbons, are present, so that the unsaponifiable matter in such oils does not represent the sterol content.

The Biological Synthesis of Sterols.—It is self-evident that synthesis of the vegetable sterols must take place in the plant kingdom. It is only recently that Channon,[67] and Randles and Knudson[68] have shown that cholesterol could be synthesized in the animal body. Randles and Knudson placed young rats on a diet free from cholesterol and from all other sterols, at least insofar as was indicated by the absence of the Liebermann-Burchard reaction. These animals were allowed to reproduce and their young were continued on a cholesterol-free diet. An analysis of the adult rat which had been born and raised on a cholesterol-free diet showed that the tissues contained many times the cholesterol which was present in the animal at birth, and indicated rather quantitatively that the animal organism can synthesize the sterols which are characteristic of its tissues.

THE ANALYSIS OF FATS AND OILS.—(Cf. Association of Official Agricultural Chemists,[69] Holland, Reed, and Buckley,[70] Methods for the Sampling and Analysis of Commercial Fats and Oils,[71] and Abderhalden.[72])

A. The Extraction of the Fats or Oils from the Plant or Animal Tissues.—In order to completely extract the fats or oils from the tissues, it is necessary that the tissues be dried, and *the drying must take place without oxidation of the fat or oil.* Various drying methods have been proposed, *i.e.*, tissues have been dried by heat in an air oven, or by heat *in vacuo*, or by heat in a neutral gas, such as nitrogen. Drying by heat

[66] Ball, C. D., Jr., A Study of Wheat Oil, *Cereal Chem.*, 3 : 19–39 (1926).

[67] Channon, H. J., Cholesterol Synthesis in the Animal Body, *Biochem. J.*, 19 : 424–432 (1925).

[68] Randles, F. S., and Knudson, A., Studies on Cholesterol. I. Synthesis of Cholesterol in the Animal Body, *J. Biol. Chem.*, 66 : 459–466 (1925).

[69] Association of Official Agricultural Chemists, Official and Tentative Methods of Analysis, Second Edition, Washington, D. C. (1925).

[70] Holland, E. B., Reed, J. C., and Buckley, J. P., Improved Methods for Fat Analysis, Bull. No. 166, Massachusetts Agr. Exp. Sta. (1915).

[71] Tentative Standard Methods for the Sampling and Analysis of Commercial Fats and Oils, *J. Ind. Eng. Chem.*, 10 : 315–320 (1918).

[72] Abderhalden, E., *Handb. biol. Arbeitsmeth.*, Abt. I, Chemische Methoden, Teil 6, Lipoide, Säuren, Cyclosen (1925).

in air is not to be recommended, and whenever possible the drying should be done *in vacuo* or in the presence of a neutral gas. In some instances, it has been found desirable to dry material by adding absolute alcohol to abstract the water and, at a low temperature, distilling off the dilute alcohol so formed. In other instances, anhydrous calcium sulfate (plaster of Paris) or anhydrous sodium sulfate is used to combine with the water, the water being bound in the form of water of crystallization. In still other instances, notably in the case of meat samples, the material has been frozen, and then the frozen material dried *in vacuo* over sulfuric acid, the desiccator being kept at a temperature below zero. Meat can be dried in this way with practically no oxidation of the fats, the meat sample retaining almost its original size and shape.

Following the drying procedure, the fat or oil is extracted by some appropriate solvent. In general, dry neutral ether is chosen as the solvent, although chloroform or carbon tetrachloride has been used by some investigators. In the event that the material contains a high percentage of protein, it is sometimes necessary to follow the original ether extraction by extraction with absolute alcohol, and this in turn by a second ether extraction. This is notably the case where fat is adsorbed upon protein surfaces or where fats or fat-like compounds are combined with proteins to form the so-called lecithoproteins or lipoproteins. Following extraction, the solvent is removed by evaporation at low temperature, leaving behind the sample of fat or oil for examination.

B. Physical Properties Which May Be Determined on the Fat or Oil Samples.—A number of physical properties have been used to characterize fats and oils. These include:

1. The specific gravity, which may be obtained by the use of the hydrometer or preferably a pycnometer or a Westphal balance.

2. The melting point, which is obtained by suspending a disk of the solid fat approximately midway in an aqueous solution which has been adjusted so as to have approximately the same specific gravity as the fat. This solution is then slowly heated, and the point is noted at which the disk of fat " rounds up " into a globule. This so-called melting point is more or less indefinite, due to the fact that a fat is composed of a number of substances having different melting points. Accordingly it is seldom worthwhile to undertake the rather tedious determination of the so-called melting point.

3. The Titre test, *i.e.*, the temperature at which the fatty acids, which have been liberated from the fat after the fat has been saponified by alkali, resolidify.

4. The refractive index, taken either with an Abbé butyro refractometer or a dipping refractometer. The refractive index is a very

important and valuable index of the composition and purity of a fat or oil.

5. The optical rotation may be taken, and in some instances gives important data.

6. The viscosity, which is again an important criterion, is very largely used in the industry. The Saybolt or the MacMichael viscosimeters are more commonly employed.

7. The specific heat: In the event that the fat or oil is to be used for purposes of lubrication, it is sometimes desirable to obtain the value for the specific heat (cf. Wesson and Gaylord[73]).

C. Color Reactions.—Numerous color reactions have been described, by which an oil from one plant or animal source may be distinguished from similar oils from other plant or animal sources. Gill,[74] however, points out that color reactions can be regarded only as *circumstantial evidence*, inasmuch as they depend upon impurities carried along into the fat or oil from its natural source. The glycerides of one plant or animal species are, in general, not different from those of other species of plants or animals. Accordingly there is no color test which will distinguish a pure fat or oil from other fats and oils.

Lewkowitsch[75] likewise has a very poor opinion of color tests as indicating the original source of an oil.

D. Qualitative Tests for Particular Classes of Fats.—1. As already noted, oleic acid is transformed into its isomer, eläidic acid, by treatment with nitric oxide. Accordingly the *eläidin test* is a qualitative test for the presence of oleins. An oil containing a large percentage of olein becomes a solid fat on treatment with nitric oxide.

2. The linoleic and linolenic acids of the drying oils, when treated with bromine, form characteristic tetra bromides and hexabromides (*vide infra*). This reaction has given rise to the so-called *hexabromide test* for drying oils. This point will be referred to later.

E. Chemical Methods.—A number of chemical methods have been proposed for the characterization of fats and oils.

1. *The Acid Number.*—The acid number may be defined as the milligrams of potassium hydroxide necessary to neutralize the free fatty acids present in one gram of a fat, oil, or wax.

2. *The Iodine Absorption Number.*—This may be defined as the

[73] Wesson, D., and Gaylord, H. P., Specific Heat of Fats and Oils, *Cotton Oil Press*, 2 : 40–41 (1918).

[74] Gill, A. H., Color Tests for Oils—Palm Oil, *J. Ind. Eng. Chem.*, 9 : 136–139 (1917).

[75] Lewkowitsch, J., Chemical Technology and Analysis of Oils, Fats, and Waxes, Fourth Edition, 3 Vols., Macmillan and Company, London (1909).

number of centigrams of iodine taken up by 1 gram of fat. In other words, it may be expressed as the percentage of iodine taken up by 1 gram of fat.

3. *The Saponification Number or the Koettstorfer Number.*—This is the milligrams of potassium hydroxide necessary to saponify 1 gram of a fat or oil.

4. *The Total Fatty Acids.*—Five grams of the fat or oil are saponified, and the fatty acids precipitated as a lead soap, using lead acetate solution. The lead soaps are washed and then decomposed by shaking with 1 : 5 hydrochloric acid, liberating the free fatty acids. These are dissolved in anhydrous ether. The ethereal solution is dried, and an aliquot of the solution evaporated, and the residue weighed.

5. *The Reichert-Meissl Number.*—This is the number of cubic centimeters of 0.1 N potassium hydroxide required to neutralize the volatile acids from 5 grams of a saponified fat or oil.

6. *The Hehner Number.*—This is the weight of the non-volatile (insoluble) fatty acids yielded by 5 grams of a saponified fat or oil.

7. *The Polenske Number.*—This is the number of cubic centimeters of 0.1 N potassium hydroxide required to neutralize the non-volatile fatty acids obtained from 5 grams of a saponified fat or oil. The residue remaining in the flask from the Reichert-Meissl determination is usually used to determine the Polenske number.

8. *The Acetyl Number.*—This is the milligrams of potassium hydroxide necessary to combine with the acetic acid liberated by the saponification of 1 gram of acetylated fat or oil.

F. The Separation, Identification, and Estimation of the Individual Constituents of an Oil, Fat or Wax.—Such a study constitutes in reality a research problem (cf. Baughman and Jamieson,[76, 77] and Ball[78]). In general, one may expect to go through somewhat the following procedure, although no definite technic can be given which should be followed irrespective of the nature of the material under investigation.

1. The fat or oil is saponified, and the saponification is followed by drying the soaps and removing the non-saponifiable residue by extracting the dry soaps with anhydrous ether. The fatty acids are then set free from the soaps and separated by distillation into the volatile fatty acids and the non-volatile fatty acids. Dry and preserve the solid non-volatile acids in petroleum ether, and use as noted under (4).

[76] Baughman, W. F., and Jamieson, G. S., The Composition of Hubbard Squash Seed Oil, *J. Am. Chem. Soc.*, 42 : 152–157 (1920).

[77] Jamieson, G. S., and Baughman, W. F., Okra Seed Oil, *J. Am. Chem. Soc.* 42 : 166–170 (1920).

[78] Ball, C. D., Jr., A Study of Wheat Oil, *Cereal Chem.*, 3 : 19–39 (1926).

2. The mean molecular weight of the volatile and the non-volatile fatty acids is obtained by titrating an aliquot with standard alkali.

3. The volatile fatty acids are separated into fractions as follows: The soaps are prepared, and these are treated with 20 per cent sulfuric acid. (*a*) The caproic and the higher acids separate as an oil which is removed. (*b*) The remaining solution is saturated with sodium chloride, butyric acid separating as an oil. (*c*) The residual salt solution is extracted with ether to remove any of the lower fatty acids which may be present. The three fractions, (*a*), (*b*), and (*c*), of the volatile fatty acids which were obtained are then converted into silver, calcium or barium salts. These salts are analyzed and from the percentage of silver, calcium, or barium which they contain, the probable composition of the fractions is determined.

4. The non-volatile fatty acids may be separated by any one of three methods, (*a*) the lead salt-ether method or (*b*) the fractional precipitation of the barium or magnesium salts, or (*c*) the fractional distillation of the esters of the acids *in vacuo*.

The lead salts of the *saturated* fatty acids are insoluble in ether, whereas the lead salts of the *unsaturated* fatty acids are soluble in ether. Accordingly the lead salt-ether method is, in general, used to separate the higher saturated fatty acids from the unsaturated fatty acids, although both the lead salt-ether method and the fractional precipitation or fractional distillation may be necessary (cf. Holland, Reed, and Buckley,[79] and Holland and Buckley[80]).

Lewis[81] has suggested a new method based upon the formation of hydroxamic acids when hydroxylamine is added to a mixture of fatty acids.

$$R—COOH + NH_2OH = R—CO—NH—OH + H_2O.$$

He notes that the sodium salts of stearohydroxamic acid and palmitohydroxamic acid are insoluble in alcohol, whereas the corresponding sodium salt of oleohydroxamic acid is freely soluble. The differences are so marked that Lewis states a quantitative separation is possible. The sodium salts of the saturated fatty acids are insoluble when the chain contains twelve or more carbon atoms, and soluble for acids con-

[79] Holland, E. B., Reed, J. C., and Buckley, J. P., Determination of Stearic Acid in Butter Fat, *J. Agr. Res.*, 6 : 101–113 (1916).

[80] Holland, E. B., and Buckley, J. P., Determination of Fatty Acids in Butter Fat: I. *J. Agr. Res.*, 12 : 719–732 (1918).

[81] Lewis, A. H., The Separation of Fatty Acids, *Biochem. J.*, 20 : 1356–1363 (1926).

taining eight or less carbon atoms. Lauric acid (C_{12}) forms a somewhat soluble sodium hydroxamic salt.

5. The liquid, unsaturated fatty acids may be separated by means of their brom derivatives or by means of their oxidation products.

a. *Separation by Means of Brom Derivatives.*—The unsaturated fatty acids are brominated in glacial acetic acid. Organic solvents are then used to separate the bromine compounds into groups showing different solubilities. The oleic group (one double bond) of fatty acids yields dibrom derivatives which are soluble in petroleum ether. The linoleic group (two double bonds) yields tetrabrom derivatives, insoluble in petroleum ether but soluble in ethyl ether. The linolenic group (three double bonds) yields hexabrom derivatives, insoluble in petroleum ether, insoluble in ethyl ether, but soluble in hot benzene.

b. *Separation by Means of Oxidation Products.*—The separation of the liquid, unsaturated fatty acids through their oxidation products is carried out by oxidizing the fatty acids to their corresponding hydroxy acids. The linolenic series yields hexahydroxystearic acids which are soluble in cold water. The linoleic series yields tetrahydroxystearic acids, soluble only in large quantities of boiling water. The oleic series yields dihydroxystearic acids, insoluble in hot water, but soluble in ethyl ether. The influence of polar hydroxyl groups is very marked in the above solubility series.

6. The solid, saturated fatty acids are separated by the fractional distillation of their methyl and ethyl esters. In many instances, the esters have to be refractionated a number of times, providing any considerable number of the higher saturated fatty acids are present. The various fractions are then converted into their silver salts. These are crystallized and analyzed for silver as a test for the purity of the various fractions.

7. The glycerol content of the liquor from which the fatty acids were removed is determined by some appropriate method.

8. The non-saponifiable residue is further studied for the presence of the higher alcohols, including the sterols. The methods used are largely those of fractional crystallization or fractional distillation, combined with the formation of chemical derivatives which may serve for identification purposes.

CHAPTER XXXII

THE LIPIDES

MacLean[1] has presented an excellent discussion of this group of compounds. The lipides are substances of a fat-like nature, yielding on hydrolysis fatty acids or derivatives of fatty acids and containing in the molecule either nitrogen or phosphorus and nitrogen.

Our knowledge of the organic phosphorus compounds occurring in both plants and animals is still very limited and incomplete (cf. Plimmer[2]). Five groups of organic phosphorus compounds occur in nature.

1. *Phytin* or *phytic acid,* in which phosphoric acid is combined with inositol as inositol phosphoric acids, phytin being the calcium-magnesium salt of inositol phosphoric acid.[3-11] Inositol is hexahydroxyhexahydrobenzene, and occurs very widely distributed throughout the plant kingdom, notably in the pericarp of seeds. The phosphoric acid combined with the inositol probably functions as a reserve phosphorus supply.

[1] MacLean, H., Lecithin and Allied Substances, The Lipins, Longmans, Green and Company, London (1918).

[2] Plimmer, R. H. A., The Metabolism of Organic Phosphorus Compounds. Their Hydrolysis by the Action of Enzymes, *Biochem. J.*, 7 : 43–71 (1913).

[3] Anderson, R. J., Phytin and Phosphoric Acid Esters of Inosite, *Tech. Bull.* No. 19, New York Agr. Exp. Sta. (1912).

[4] Anderson, R. J., Phytin and Pyrophosphoric Acid Esters of Inosite. II, *Tech. Bull.* No. 21, New York Agr. Exp. Sta. (1912).

[5] Anderson, R. J., The Organic-Phosphoric Acid Compound of Wheat Bran, *Tech. Bull.* No. 22, New York Agr. Exp. Sta. (1912).

[6] Anderson, R. J., The Organic Phosphoric Acid of Cottonseed Meal, *Tech. Bull.* No. 25, New York Agr. Exp. Sta. (1912).

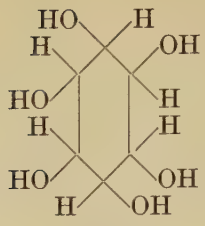
[7] Anderson, R. J., A Contribution to the Chemistry of Phytin, *Tech. Bull.* No. 32, New York Agr. Exp. Sta. (1914).

[8] Anderson, R. J., Organic Phosphoric Acids of Wheat Bran, *Tech. Bull.* No. 36, New York Agr. Exp. Sta. (1914).

[9] Anderson, R. J., Concerning the Organic Phosphorus Compound of Wheat Bran and the Hydrolysis of Phytin, *Tech. Bull.* No. 40, New York Agr. Exp. Sta. (1915).

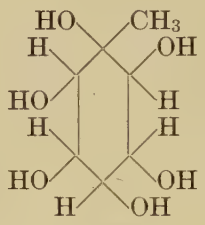
[10] Anderson, R. J., Concerning Inosite Phosphoric Acids, *Tech. Bull.* No. 79, New York Agr. Exp. Sta. (1920).

[11] Averill, H. P., and King, C. G., The Phytin Content of Foodstuffs, *J. Am. Chem. Soc.*, 48 : 724–728 (1926).



Inositol

The inositol itself has become of added interest following the observation of Eastcott[12] that inositol is bios I, a substance necessary for the normal reproduction of the yeast cell (cf. Wildiers[13]). A compound analogous to inositol, but a derivative of hexahydrotoluene instead of hexahydrobenzene, is mytilit, isolated by Ackermann[14] from the mussel, *Mytilus edulis*. Its physiological importance is unknown.



Mytilit

2. *The phospholipides* (the phosphatides of Thudichum), of which lecithin is the best known example. Here the phosphorus is in ester combination with glycerol, as glycerol phosphoric acid. Phospholipides occur in both plants and animals, but in general in larger amounts in animal cells.

3. *The hexose phosphates*, according to Harden,[15] and Harden and Young,[16] are intermediary products in the alcoholic fermentation by yeast.

[12] Eastcott, E. V., Wildiers' Bios. The Isolation and Identification of "Bios I," *J. Phys. Chem.*, 32 : 1094–1111 (1928).

[13] Wildiers, E., Nouvelle substance indispensable au développement de la levûre. *La Cellule*, 18 : 313–333 (1901).

[14] Ackermann, D., Über den Mytilit, eine natürlich vorkommende Cyclose, *Ber.*, 54 : 1938–1943 (1921).

[15] Harden, A., Alcoholic Fermentation, Longmans, Green and Company, London (1911).

[16] Harden, A., and Young, W. J., The Alcoholic Ferment of Yeast-Juice, *Proc. Roy. Soc.*, B., 77 : 405–420 (1906).

4. *Nucleic acids* have already been discussed. Here phosphoric acid is combined with carbohydrates and purine and pyrimidine bases.

5. *The phosphoproteins* have likewise already been discussed. The best known examples are casein and vitellin. The phosphorus is present in the protein molecule presumably as phosphoric acid, but the nature of the linkage is unknown. The phosphoproteins, insofar as we are aware, occur only in the animal kingdom.

The Phospholipides.—This group may be further subdivided into (1) the *monoaminomonophospholipides*, having a ratio of one atom of nitrogen to one atom of phosphorus, of which lecithin and cephalin are the best known examples, and (2) the *diaminomonophospholipides*, having the ratio of two nitrogen atoms to one atom of phosphorus, and having as a typical example, sphingomyelin.

The work in the field of the lipides dates from the publication by Thudichum, in 1874, of a paper entitled, " Researches on the Chemical Constitution of the Brain."[17] Thudichum continued to publish in this difficult field of chemical research for a number of years. Of recent years many workers have contributed to this field, chief among whom should be noted P. A. Levene and O. Rosenheim.

Probably no field of biological chemistry offers more difficulties than does the study of the separation and identification of the chemical structure of the lipides, comprising such a large fraction of the nervous tissue.

The phospholipides upon hydrolysis yield phosphoric acid, various fatty acids, and basic substances, such as choline (trimethyl-β-ethyl alcohol ammonium hydroxide) or β-aminoethyl alcohol. The phospholipides are soluble in ordinary fat solvents but are *insoluble in and precipitated by acetone.* They form crystallizable addition products with platinic chloride and with cadmium chloride. Many are hydrophilic, even hygroscopic, and imbibe water to form colloidal sols.

Lecithin, one of the best known examples, is an ester of glycerol. Ordinary commercial lecithin is probably a mixture of two substances, one containing the base, choline, the other aminoethyl alcohol. Formulae (*A*) and (*B*) have been generally ascribed to lecithin, with (*B*) being accepted as the more probable form.

However, Grün and Limpächer[18] have synthesized lecithin by first treating distearin (or some other diglyceride) with one molecule of phos-

[17] Thudichum, J. L. W., Researches on the Chemical Constitution of the Brain, Report of the Medical Officer of the Privy Council and Local Government Board, New Series, 3 : 113–247 (1874).

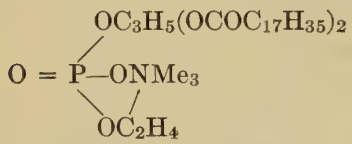
[18] Grün, A., and Limpächer, R., Synthese der Lecithine, I, II, *Ber.* 59 : 1350–1360 (1926); 60 : 147–150 (1927).

CH_2—O—fatty acid
|
CH—O—fatty acid
|
CH_2—O—phosphoric acid—O—$N(CH_3)_3$—CH_2—CH_2—OH
(*A*)

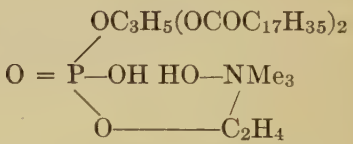
and

CH_2—O—fatty acid
|
CH—O—fatty acid
|
CH_2—O—phosphoric acid—O—CH_2—CH_2—$N(CH_3)_3$—OH
(*B*)

phorus pentoxide and then treating the product with two molecules of bicarbonate of choline or some other salt of choline. The lecithin was isolated by neutralizing its benzene solution with alcoholic alkali, thus separating choline-phosphoric acid, and by precipitating with acetone, thereby separating distearin. Lecithin was identified by analysis and by its physical and chemical properties. The following formulae are assigned to it:



Anhydride form of lecithin



Hydrated form of lecithin

It will be noted that the anhydride formula has both the hydroxyl group of the ethyl alcohol and the hydroxyl group of the ammonium hydroxide united with the phosphoric acid.

The fatty acids of the lecithin may be either oleic, stearic, or palmitic. Thudichum states that all true lecithins contain one unsaturated fatty acid, usually oleic. It is obvious, if the two fatty acids are different, that stereoisomeric compounds are possible.

Lecithin is optically active, indicating that the molecule is asymmetric and that the middle carbon atom of the glycerol is optically active, *e.g.*, the molecule is of type (*A*) rather than of type (*B*).

CH_2—O—fatty acid
|
CH—O—fatty acid
|
CH_2—O—phosphoric acid
(*A*)

rather than

CH_2—O—fatty acid
|
CH—O—phosphoric acid
|
CH_2—O—fatty acid
(*B*)

That the optical activity is not connected with the fatty acid radicals is indicated by the fact that the glycerol phosphoric acid which can be prepared from lecithin is likewise optically active.

Lecithin may be separated from the associated fats by dialyzing the fats through a thin rubber membrane. The material extracted by a fat solvent is dissolved in ether and placed inside of a thin rubber dialyzing bag and dialyzed against ether. The fats will rather rapidly pass through the rubber membrane, but the phospholipides will remain behind. Practically pure lecithin can thus be prepared from butterfat (cf. Gies[19]).

Lecithin is a yellowish-white, waxy solid, quite hygroscopic, and absorbs oxygen from the air, becoming brown. It is an excellent emulsifying agent. It is readily hydrolyzed by lipase. *It is a constituent of all cells*, and consequently must possess a very important function in life processes. It has been suggested that one of its functions is in relation to the permeability of the cell membrane. It can be synthesized by animals which are on a lecithin-free diet; consequently we are not dependent upon our food supply for our lecithin.

Cephalin or kephalin is usually associated with lecithin. It is distinguished from lecithin by the fact that it is *insoluble in alcohol.* The decomposition products which have been isolated indicate that cephalin is composed of phosphoric acid, glycerol, aminoethyl alcohol, saturated fatty acids (usually stearic acid), and unsaturated fatty acids.

Grün and Limpächer[20] claim to have synthesized cephalin, using a procedure analogous to that which they used in synthesizing lecithin. The compound which they synthesized, however, contained two molecules of stearic acid, whereas Levene and Rolf[21] isolated from cephalin, oleic acid and arachidonic acid, as well as stearic acid.

Sphingomyelin yields as decomposition products phosphoric acid, lignoceric acid (cf. Levene[22]), cerebronic acid (cf. Levene and Taylor[23]), and two bases, choline and sphingosine ($C_{17}H_{35}NO_2$) (cf. Levene,[24] and

[19] Gies, W. J., Studies of Diffusion through Rubber Membranes. I. Preliminary Observations on the Diffusibility of Lipins and Lipin-Soluble Substances, *Biochem. Bull.*, 2 : 55–63 (1912); Rosenbloom, J., II. Diffusibility of Lipins from Ether through Rubber Membranes into Ether, *Biochem. Bull.*, 2 : 64–69 (1912).

[20] Grün, A., and Limpächer, R., Synthese der Kephaline, *Ber.*, 60 : 151–156 (1927).

[21] Levene, P. A., and Rolf, I. P., Unsaturated Fatty Acids of Brain Cephalins, *J. Biol. Chem.*, 54 : 91–100 (1922).

[22] Levene, P. A., Sphingomyelin, *J. Biol. Chem.*, 15 : 153–154 (1913).

[23] Levene, P. A., and Taylor, F. A., The Synthesis of α-Hydroxyisopentacosanic Acid and Its Bearing on the Structure of Cerebronic Acid, *J. Biol. Chem.*, 52 : 227–240 (1922).

[24] Levene, P. A., Sphingomyelin, II and III, *J. Biol. Chem.*, 18 : 453–462 (1914); 24 : 69–89 (1916).

Rosenheim and MacLean[25]). Sphingosine appears to be an unsaturated diatomic amino alcohol, possessing apparently the structure,

$$C_{12}H_{25}CH{=}CH{-}CHOH{-}CHOH{-}CH_2NH_2.$$

Sphingomyelin is a crystallizable non-hygroscopic substance characteristic of nervous tissue and is relatively stable on exposure to light and air.

Sulfur-Containing Phospholipides.—Fränkel and Karpfen[26] note that Thudichum isolated a sulfur-containing phospholipide from brain tissue which he called cerebrosulfatide. This was further studied by Koch[27] who observed that the compound contained equal molecular amounts of sulfuric acid and phosphoric acid. Fränkel and Karpfen have recently presented a further study of this compound. They give the empirical formula as $C_{101}H_{152}N_3PSO_{26}$, having a ratio of S : P : N of 1 : 1 : 3, all of the nitrogen being in the form of amino groups. They have identified as hydrolytic products, phosphoric acid, sulfuric acid, glycerol, and aminoethyl alcohol. All of the nitrogen was present in the form of the aminoethyl alcohol, choline and sphingosine being completely absent. The only fatty acid which appeared to be present was a hitherto unknown hydroxy acid, α-hydroxy-*n*-decanic acid, $C_{10}H_{20}O_3$.

Fränkel and Gilbert[28] had previously isolated from human brain substance another phospholipide containing sulfur, the compound having the formula, $C_{93}H_{191}N_3SPO_{18}$. The hydrolytic products of this compound were phosphoric acid, sulfuric acid, glycerol, aminoethyl alcohol, and cerebronic acid. Just how these various decomposition products are linked in the lipides remains to be determined.

The Glucolipides or Cerebrosides (*Thudichum*).—The glucolipides occur chiefly in the brain and other nervous tissues, although they are not wholly restricted to nervous tissues. As decomposition products they yield a sugar (galactose), a base (sphingosine), and a fatty acid. They do not contain phosphorus. When pure, they form a white powder with a crystalline or waxy appearance. They usually occur asso-

[25] Rosenheim, O., and MacLean, H., Lignoceric Acid from "Carnaubon," *Biochem. J.*, 9 : 102–109 (1915).

[26] Fränkel, S., and Karpfen, O., Über die Hypohirnsäure, *Biochem. Z.*, 157 : 414–424 (1925).

[27] Koch, W., Zur Kenntnis der Schwefelverbindungen des Nervensystems. II. Über ein Sulfatid aus Nervensubstanz, *Z. physiol. Chem.*, 70 : 94–97 (1910).

[28] Fränkel, S., and Gilbert, O., Über die Darstellung von Phosphorsulfatiden aus Gehirn, *Biochem., Z.* 124 : 206–215 (1921).

ciated with phospholipides, from which, in some instances, they are separated only with difficulty. Because of the presence of the carbohydrate radical, glucolipides are optically active. The color tests which they yield appear to be due to the carbohydrate group.

Pryde and Humphreys[29] have investigated the nature of the sugar residue of the glucolipides, taking care to methylate the galactose before the compound was hydrolyzed. The methyl galactose so obtained proved to be the amylene oxide or the δ-oxide form of galactose. They note that this is the stable form of galactose and that possibly the carbohydrate radical of the glucolipides may be looked upon as existing in the same form as that in which glucose is present in the naturally-occurring glucosides.

Among the glucolipides which are better known are:

1. *Phrenosin* ($C_{48}H_{93}NO_9$) which can be obtained in the form of a white powder, the crystals having more or less the general appearance of cholesterol. Phrenosin very readily forms "liquid crystals."

Levene and West[30] described certain derivatives of phrenosin, and Takaki[31] has noted that phrenosin will combine with tetanus toxin. On acid hydrolysis phrenosin yields cerebronic acid, sphingosine, and *d*-galactose. It is not hydrolyzed by pancreatic lipase or by emulsin. The amino group of sphingosine is combined in the molecule, inasmuch as no nitrogen is liberated in the Van Slyke apparatus.

Cerebronic acid, $C_{25}H_{50}O_3$, is a hydroxy acid which has been investigated by Levene and West.[32, 33] The exact configuration of the molecule still remains to be determined.

2. *Kerasin* ($C_{47}H_{91}NO_8 \cdot H_2O$) (cf. Rosenheim[34]) is a compound having properties very similar to those of phrenosin, and accompanies phrenosin in nervous tissue. The "protagon" of the older workers appears to be, in the light of recent investigations, a mixture of phrenosin and kerasin. On acid hydrolysis kerasin yields lignoceric acid ($C_{24}H_{48}O_2$), sphingosine, and *d*-galactose.

[29] Pryde, J., and Humphreys, R. W., The Nature of the Sugar Residue of the Cerebrosides of Ox-Brain, *Biochem. J.*, 20 : 825–828 (1926).

[30] Levene, P. A., and West, C. J., Cerebrosides. IV. Cerasin, *J. Biol. Chem.*, 31 : 635–647 (1917).

[31] Takaki, K., Über Tetanusgift bindende Bestandteile des Gehirns, *Beitr. chem. Physiol.*, 11 : 288–303 (1908).

[32] Levene, P. A., and West, C. J., On Cerebronic Acid, *J. Biol. Chem.*, 14 : 257–265 (1913).

[33] Levene, P. A., and West, C. J., On Cerebronic Acid. IV. On the Constitution of Lignoceric Acid, *J. Biol. Chem.*, 18 : 477–480 (1914).

[34] Rosenheim, O., The Galactosides of the Brain. IV. The Constitution of Phrenosin and Kerasin, *Biochem. J.*, 10 : 142–159 (1916).

The Plant Lipides.—Our present knowledge of the plant lipides is very limited. We do not know with certainty whether or not plant and animal lipides are identical in chemical constitution. The chief studies of plant lipides have centered around the phospholipides, inasmuch as these can be more readily followed in the course of fractionation by studying the percentage of the phosphorus. Dried seeds of legumes may contain as much as 1.5 per cent of phospholipides; the cereal grains usually contain approximately 0.5 per cent.

Carbohydrates appear to be more generally present in the plant lipides than is the case with animal lipides. Plant lecithin entirely free from carbohydrate has not as yet been obtained, although the hydrolysis products of plant lecithin, *i.e.*, glycerol, phosphoric acid, choline, and aminoethyl alcohol, are the same as are yielded by the animal lecithins. Levene and Rolf[35] have investigated the plant phospholipides of the soy bean. The lecithin fraction yielded on hydrolysis stearic and palmitic acids, oleic acid, and acids belonging to the linoleic and linolenic series. No unsaturated acid containing more than eighteen carbon atoms could be isolated. They likewise found, in addition to lecithin, a compound having the properties of cephalin, which appears to be similar to, if not identical with, the cephalin isolated from animal sources. The lecithin likewise was found to contain a carbohydrate which was identified as a pentose, on the basis of color reactions with orcinol. Aminoethyl alcohol was readily isolated as the aurichloride.

In regard to the biological functions of the lipides in general, we only know that the phospholipides are essential constituents of all cells, which indicates that they must be essential for the life process (cf. Levene[36]). The fact that they are all good emulsifying agents suggests the possible function of maintaining the proper colloidality of protoplasm. They are all more or less toxic if given in larger doses than can be readily assimilated by the tissues.

Insofar as the author is aware, carbohydrates do not occur in nature esterified with fatty acids, except in the case of the glucolipides. Bloor,[37-39] however, has synthesized many carbohydrate derivatives

[35] Levene, P. A., and Rolf, I. P., Plant Phosphatides. I. Lecithin and Cephalin of the Soy Bean, II. Lecithin, Cephalin, and So Called Cuorin of the Soy Bean, *J. Biol. Chem.*, 62 : 759–766 (1925); 68 : 285–293 (1926).

[36] Levene, P. A., The Chemical Individuality of Tissue Elements and Its Biological Significance, *J. Am. Chem. Soc.*, 39 : 828–837 (1917).

[37] Bloor, W. R., Carbohydrate Esters of the Higher Fatty Acids. II. Mannite Esters of Stearic Acid, *J. Biol. Chem.*, 11 : 141–159 (1912).

[38] Bloor, W. R., On Fat Absorption, *J. Biol. Chem.*, 11 : 429–434 (1912).

[39] Bloor, W. R., Fatty Acid Esters of Glucose, Original Communications to the Eighth Internat. Congress of Applied Chem., 19 : 29–36 (1912).

which resemble fats, by substituting sugars as alcohols in place of glycerol. He finds that when these substances are fed to dogs, they are well utilized, but that they must first be hydrolyzed by lipase in the intestinal tract before they are absorbed. It will be noted that these synthetic compounds are hydrolyzable by lipase, whereas phrenosin is not, indicating a somewhat different linkage in phrenosin from that which exists in Bloor's synthetic compounds. Only recently has it become generally recognized that fats and lipides must be hydrolyzed before absorption from the intestinal tract takes place. It was formerly thought that such compounds passed through the mucosa of the intestine unhydrolyzed in the form of a fine emulsion. We now know that in order to metabolize a fat, it must first be made water-soluble, *i.e.*, be hydrolyzed to soaps and glycerol, and that emulsification is not sufficient (cf. Bloor,[40, 41] and Smedley and Lubrzynska[42]).

[40] Bloor, W. R., On Fat Absorption. II. Absorption of Fat-Like Substances Other than Fats. III. Changes in Fat During Absorption, *J. Biol. Chem.*, 15 : 105–117 (1913); 16 : 517–529 (1914).

[41] Bloor, W. R., The Distribution of the Lipoids ("Fat") in Human Blood, *J. Biol. Chem.*, 25 : 577–599 (1916).

[42] Smedley, I., and Lubrzynska, E., The Biochemical Synthesis of the Fatty Acids, *Biochem. J.*, 7 : 364–374 (1913).

CHAPTER XXXIII

ESSENTIAL OILS

The essential oils may be defined as "those compounds in plants which are volatile with steam and usually separate as an oily layer in the distillate." They are present to a small extent in most plants and may be present to a very considerable extent in certain families or groups of plants. Some essential oils are of interest only from the scientific standpoint, involving the identification or the preparation of rare organic compounds. Others, such as oils of wintergreen, clove, cinnamon, bergamot, attar of roses, lemon and orange oils, camphor, cedar oil, pine oil, eucalyptus oil, turpentine, etc., are of very considerable commercial importance.

In certain plants essential oils may occur in all of the tissues. This is notably true of the conifers. In the rose they occur in appreciable amounts only in the petals, in cinnamon only in the bark and leaves, in the orange in the petals of the flowers and the skin of the fruit, in the nutmeg chiefly in the fruit, and in the camphor tree both in the leaves and throughout the entire woody tissue.

The function of essential oils in the plant kingdom is unknown. Here again, various suggestions have been made, as in the case of the tannins, that they may be waste products, or protection against injury or the invasion of fungi, and that they may provide an odor which will attract insects so as to favor pollination. Very closely allied species may differ greatly in their content of essential oils and in the chemical constituents present in the essential oils.

Miller[1] investigated the essential oils of three species of *Pycnanthemum*, *P. tullia*, *P. incanum*, and *P. lanceolatum*. Miller notes that it is very difficult to sharply separate these three species on morphological grounds. He found, however, that the compounds present in the essential oils were widely different. Thus, the essential oil of *P. tullia* consisted of approximately 50 per cent of cineol; that from *P. incanum*

[1] Miller, E. R., A Chemical Investigation of the Volatile Oils of Some Species of the Genus *Pycnanthemum* Michx., Ph.D. thesis on file in the Library, University of Minnesota, and privately printed (1918).

consisted of approximately 90 per cent of pulegone, whereas the main fraction of the oil from *P. lanceolatum* appeared to consist of carvacrol, only approximately 5 per cent of pulegone being present. Miller accordingly suggests in his discussion that a study of the essential oils might well be undertaken in certain families as at least an assistance in determining botanical classifications.

The field of the chemistry of the essential oils presents many exceedingly difficult problems. Some oils consist almost wholly of a single compound. Other oils are mixtures containing a dozen or more compounds of the most diverse types (cf. Power[2]).

The physical methods which are used for identification of essential oils are somewhat similar to those already noted for the usual fats and oils, such as (1) optical rotation, (2) specific gravity, (3) index of refraction, and (4) boiling point range, *i.e.*, the temperature range over which an oil can be completely distilled.

The laboratory methods which are used to separate the compounds present in the mixture are in part: (cf. Parry,[3] and Semmler[4]).

1. Low temperatures, such as freezing point.—By the use of this method it is sometimes possible to crystallize out certain of the constituents.

2. Fractional distillation with steam will serve to separate an oil into the more volatile and the less volatile fractions.

3. Fractional distillation *in vacuo* of the dried oil again serves to bring about a partial separation, as does

4. Crystallization from poor solvents of certain oils and fractions of certain oils.

5. The constituents of an essential oil possessing free acidic groups may be removed by shaking the oil with sodium carbonate solution and separating the aqueous solution of the sodium salts so formed from the main bulk of the oil by means of a separatory funnel.

6. Similarly, basic compounds may be removed by shaking the oil with a dilute solution of hydrochloric acid and removing the acid solution containing the bases by means of a separatory funnel.

7. Phenols may be removed by shaking the oil with a dilute solution of sodium or potassium hydroxide.

8. Aldehydes and ketones may be removed by shaking the oil with a

[2] Power, F. B., The Distribution and Characters of Some of the Odorous Principles of Plants, *J. Ind. Eng. Chem.*, 11 : 344–352 (1919).

[3] Parry, E. J., The Chemistry of Essential Oils and Artificial Perfumes, Scott, Greenwood and Company, London (1899).

[4] Semmler, F. W., Die aetherischen Öle, 4 vols., Veit and Company, Leipzig (1906–1907).

saturated solution of sodium bisulfite, resulting in the formation of the crystalline bisulfite addition products which are non-oil-soluble.

9. By a determination of the acetyl value, the free hydroxyl groups which are present in the oil may be determined.

10. The acids which were separated by shaking with sodium hydroxide are liberated by the acidification of the solution and are converted into esters or into their silver or barium salts. The saponification number of the esters or the percentage of silver or barium in the salts provides a means of identifying the acids which are present.

11. In many instances, specific compounds which occur frequently in essential oils may be tested for by means of special color reactions.

Undoubtedly the average individual is chiefly interested in the essential oils as the basis of the perfume industry. The perfume industry is very highly developed in France. At Grasse, a city of approximately 20,000 inhabitants, the entire countryside is one flower garden. This city alone uses each year (cf. Bogert[5]) 2200 tons of orange blossoms, 1650 tons of rose petals, 1320 tons of jasmine petals, 440 tons of violets, 330 tons of tuberoses, 165 tons of carnations, 110 tons of cassie, 880 tons of mimosa branches, 66 tons of mignonette, and 55 tons of narcissus. One pound of orange blossom oil was obtained from 1000 pounds of blossoms. One pound of attar of roses required 8 tons of petals.

Three methods of extraction are employed when the oils are to be made into perfumes.

1. *Extraction with Petroleum Ether.*—This gives a greater yield, but other substances are extracted along with the true essential oils, resulting in a lower grade of perfume.

2. *Maceration of the Material with Warm Oil or Fat.*—The material is ground in the presence of added oil or fats. The fat containing the essential oil is then expressed by means of a hydraulic press, and the essential oil recovered by extracting the fat with strong alcohol. The fat (lard) is largely insoluble in the alcohol and crystallizes out on cooling, leaving an alcoholic solution of the odoriferous principles. This is a better method than (1) but still yields an inferior grade of perfume for many flowers.

3. *Cold Absorption.*—Thin layers of cold fat (40 per cent beef, 60 per cent lard) are spread about one-eighth inch thick on both sides of glass plates, and a layer of petals about two inches deep is placed above these. The layers of fat and petals are racked up one on top of the other as high as the operator can reach. After twenty-four to seventy-

[5] Bogert, M. T., The Flower and the Organic Chemist: Perfumes—Natural and Synthetic, *J. Ind. Eng. Chem.*, 14 : 359–364 (1922).

two hours (depending upon the flower being used and the quality of perfume desired), the flowers are removed and new flowers added. Finally the layers of fat are extracted with *cold alcohol*, the fat which dissolves in the alcohol is frozen out and the alcoholic solution is concentrated or bottled directly. This process yields the finest perfumes.

The essential oils as used in industrial processes or in medical practice may be obtained by three general methods:

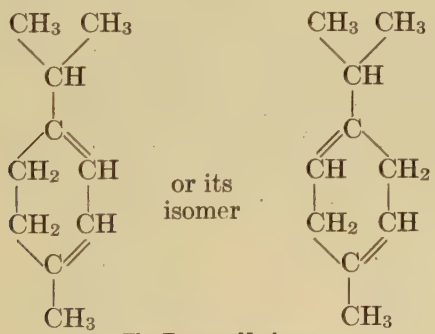
1. By some suitable solvent, such as petroleum ether, dichlor ethylene, etc., the solvent being of such a nature that it can be readily and completely removed by evaporation at a low temperature.

2. In some instances, oils may be extracted by pressure, using ground material and a hydraulic press.

3. The more general method, however, is that of steam distillation, steam from a boiler or superheated steam being passed through a mass of raw material which contains the essential oils. This steam passing through the material carries the oil over, the oil separating as a layer in the distillate (cf. Sievers[6]).

As already noted, various types of chemical compounds, such as hydrocarbons, alcohols, ketones, aldehydes, acids, esters, organic sulfides, bases, etc., may occur in essential oils. Only a few typical examples will be noted to illustrate the diversity of compounds which may be present.

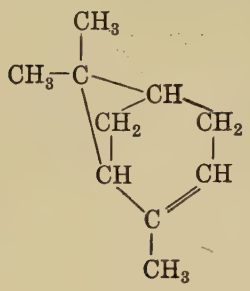
I. *Terpenes.*—These are hydrocarbons of the general formula, $C_{10}H_{16}$, and in general possess the structure,

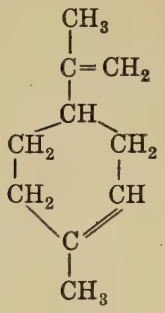


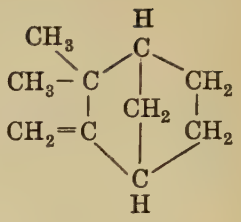
The Terpene Nucleus

Certain of the typical terpenes are pinene (from conifers), limonene (from lemon-grass oil), and camphene (from the camphor tree).

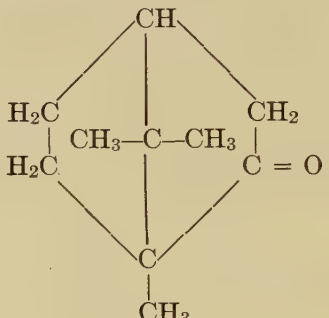
[6] Sievers, A. F., Methods of Extracting Volatile Oils from Plant Material and the Production of Such Oils in the United States, *Tech. Bull.* No. 16, U. S. Dept. Agr. (1928).


Pinene

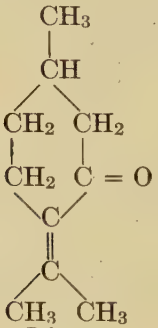

Limonene

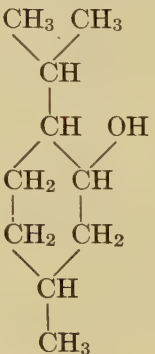

Camphene

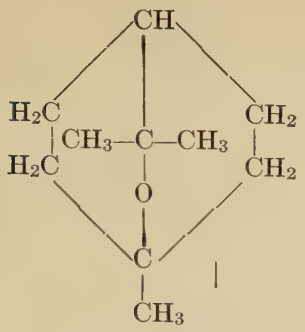
II. *Alcohols and Ketones.*—These types of compounds are very abundant in essential oils. The camphor series includes camphor, borneol, pulegone, menthol, etc.


Camphor

Camphor is obtained from the wood of the camphor tree. It has been synthesized, and the synthetic product is becoming a serious competitor of the natural product. It would appear that the camphor industry faces at the present time the same problems that the natural indigo industry faced a few decades ago.


Pulegone


Menthol

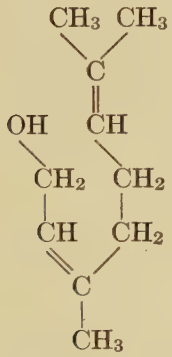


Cineol or Eucalyptol

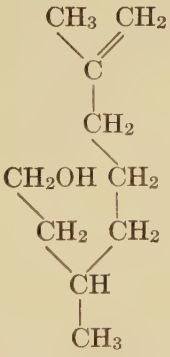
Borneol has the same structural groupings as camphor with the exception that the keto group is replaced by a secondary alcohol group.

Menthol occurs in oil of peppermint to the extent of 65 to 85 per cent, depending on the quality of the peppermint oil. The corresponding ketone, menthone, also occurs in peppermint oil. Cineol or eucalyptol is very widely distributed as a component of many of the essential oils and occurs in a very large amount in oil of eucalyptus.

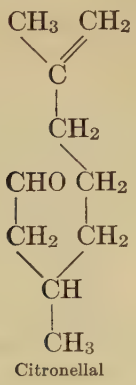
III. *Geraniol and Citronellol Group* of alcohols, ketones, and aldehydes is another important group. Here we do not have a closed ring, but the formulae may be written so as to indicate their relationship to the closed ring structures, which have already been noted.



Geraniol



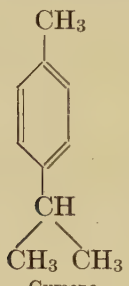
Citronellol



Citronellal

Geraniol occurs in oils of rose, geranium, sassifras, lavender, etc. Citronellal occurs in lemon skin and in orange skin oils, eucalyptus oil, lemon-grass oil, etc.

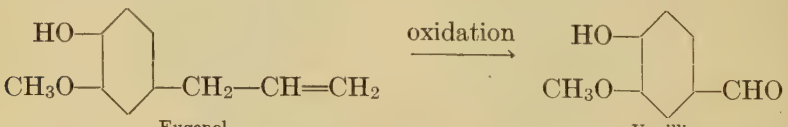
IV. *Benzene Hydrocarbons* (not terpenes) are of common occurrence, *e.g.*, cymene occurs in caraway oil, styrene in stryrax balsam.



Cymene

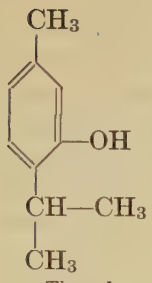
Styrene

V. *Phenols* are widely distributed in the essential oils. Thus, eugenol is the principal constituent of oil of cloves. On oxidation eugenol is converted to the corresponding aldehyde, vanillin, the flavoring principle of the vanilla bean.

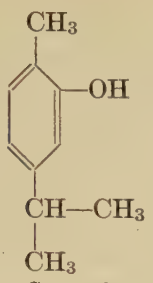


Eugenol Vanillin

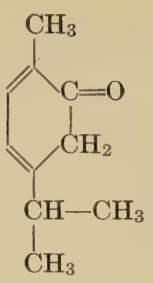
Thymol (isopropyl *m*-cresol) occurs in a number of essential oils but particularly in the oil of thyme. It is a hydroxy derivative of cymene which is likewise found in oil of thyme and in eucalyptus oil. The corresponding *o*-cresol derivative is carvacrol, occurring in many essential oils, and the corresponding ketone of carvacrol is carvone.



Thymol



Carvacrol



Carvone

VI. *Acids and esters of acids* are of common occurrence. These include benzoic acid and its esters, salicylic acid and its esters. The esters of acetic, butyric, and valeric acids are of rather common occurrence. Methyl salicylate, the methyl ester of *o*-hydroxy benzoic acid is practically the only constituent of pure oil of wintergreen and oil of birch. Ethyl acetate is the chief constituent of oil of banana. Amyl valerianate and ethyl anthranilate (the ethyl ester of *o*-amino benzoic acid) are the chief constituents of oil of apples. Amyl butyrate occurs in oil of apricots. Ethyl salicylate, ethyl butyrate, and amyl acetate occur in the fruit of the strawberry.

VII. A number of the *aliphatic alcohols* are of common occurrence. Some of these are water-soluble and do not separate with the oil but remain in the aqueous portion of the distillate from which they must be removed by extraction with a suitable non-miscible solvent or by fractional steam distillation.

VIII. *Aliphatic aldehydes* in small amounts, such as traces of formaldehyde, acetaldehyde, etc., are of rather common occurrence.

IX. *Sulfides* occur in certain oils. Thus, allyl sulfide,

$$CH_2{=}CH{-}CH_2{-}S{-}CH_2{-}CH{=}CH_2,$$

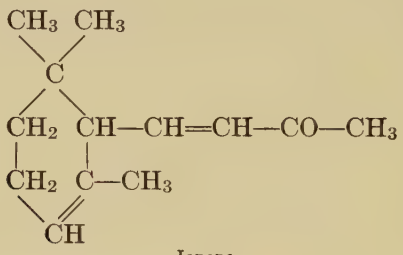
occurs in oil of garlic, and allyl thiocyanate,

$$CH_2{=}CH{-}CH_2{-}N{=}C{=}S,$$

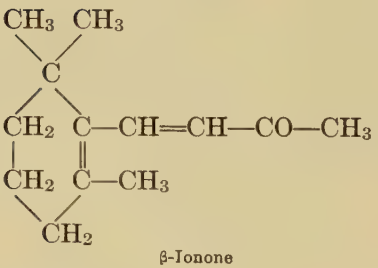
occurs in oil of mustard. Essential oils containing sulfur are especially abundant in the *Cruciferae*. In many instances the sulfur-containing radical is combined with sugars in the form of a glucoside. Thus, allyl thiocyanate occurs in mustard seed as a glucoside which must be hydrolyzed either by enzymatic action or by chemical means before the allyl thiocyanate can be distilled as an essential oil.

X. Small amounts of the *paraffin hydrocarbons*, C_nH_{2n+2}, are occasionally found in essential oils, but their occurrence is rare.

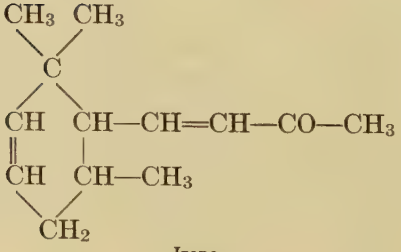
XI. Essential oils usually contain small amounts of *organic bases.* It is still a debatable question as to whether these are, in general, normal constituents of essential oils or have been derived from proteins, lipides, or other nitrogenous constituents of the plant by some decomposition process, either that of the natural autolysis of the plant material or a decomposition brought about by the methods used for preparing the essential oil. It seems probable that they are secondary decomposition products. The one possible exception is indole which occurs in extremely small amounts in the oil of orange blossoms. Indole, when present in appreciable amount, has a very disagreeable and penetrating odor. The amount which is present in orange blossom oil is, however, extremely small and in conjunction with the other constituents of the oil produces the characteristic orange blossom perfume. The synthetic perfume failed to completely simulate the natural perfume until a trace of indole had been added.



α-Ionone



β-Ionone



Irone

Most of the perfumes which are sold to the consumer represent blends which are mixtures of natural essential oils, or blends of compounds which have been synthesized in the organic laboratory. In some instances compounds have been synthesized which very closely resemble in their chemical structure the constituents occurring in the natural perfumes. Thus, α- and β-*ionone* have been prepared in the attempt to synthesize *irone*, the compound responsible for the delightful odor of violets. Irone has as yet defied synthesis. A mixture of the synthetic α- and β-ionone compounds, which differs from irone only in the position of the double bond,[7] produces a violet perfume so nearly like the natural product as to defy detection by all but the most expert of perfume connoisseurs.

[7] Hibbert, H., and Cannon, L. T., Condensation of Citral with Ketones and Synthesis of Some New Ionones, *J. Am. Chem. Soc.*, 46 : 119–130 (1924).

CHAPTER XXXIV

VITAMINS*

Vitamins are classified according to a modification of the scheme first proposed by McCollum and Kennedy,[1] *i.e.*, by the letters of the alphabet. At the present time nutrition workers recognize the existence of six vitamins which, according to the recommendations of a committee of the American Society of Biological Chemists, are designated by the letters A, B, C, D, E, and G.

It is not only possible but probable that other vitamins, perhaps many others, exist. (cf. Bezssonoff,[2] Evans and Burr,[3] Palmer and Kennedy,[4] Williams and Waterman,[5] and McCay, Bing, and Dilley.[6]) This should not be surprising in view of the complexity of life processes and the extreme differentiation exhibited in all forms of higher animals.

It has been found convenient from a chemical standpoint to classify the known vitamins according to their solubility in fat (or fat solvents) or in water. Fat-soluble and water-soluble vitamins, however, have more or less solubility in alcohol, strong alcohol favoring the fat-soluble and dilute alcohol the water-soluble vitamins, as would be expected. According to this method of classification, vitamins A, D, and E belong to the fat-soluble group, and vitamins B, C, and G to the water-soluble.

* This chapter is contributed by Dr. Leroy S. Palmer, Professor of Agricultural Biochemistry, in the University of Minnesota.

[1] McCollum, E. V., and Kennedy, C., The Dietary Factors Operating in the Production of Polyneuritis, *J. Biol. Chem.*, 24 : 491–502 (1916).

[2] Bezssonoff, N., L'effet antiscorbutique est-il dû à deux substances différentes? *Compt. rend.*, 183 : 1309–1310 (1926); *Bull. soc. chim. biol.*, 9 : 568–579 (1927).

[3] Evans, H. M., and Burr, G. O., New Dietary Deficiency with Highly Purified Diets. II. Supplementary Requirement of Diet of Pure Casein, Sucrose, and Salt, *Proc. Soc. Exp. Biol. Med.*, 25 : 41–48 (1927).

[4] Palmer, L. S., and Kennedy, C., The Fundamental Food Requirements for the Growth of the Rat. I. Growth on a Simple Diet of Purified Nutrients. II. The Effect of Variations in the Proportion and Quality of Recognized Nutrients, *J. Biol. Chem.*, 74 : 591–611; 75 : 619–659 (1927).

[5] Williams, R. R., and Waterman, R. E., The Tripartite Nature of Vitamin B, *J. Biol. Chem.*, 78 : 311–322 (1928).

[6] McCay, C. M., Bing, F. C., and Dilley, W. E., Factor H in the Nutrition of Trout, *Science*, 67 : 249–250 (1928).

No attempt will be made here to describe the historical steps through which the vitamins were discovered. It will be sufficient to point out that these fields eventually combined into what has been referred to by McCollum as the newer knowledge of nutrition. These two fields of work were first, a study of certain specific diseases, *i.e.*, beri-beri and scurvy, and second, attempts to nourish laboratory animals on synthetic diets composed of known food substances.

The term, " vitamine," from which our present word, vitamin, is derived, was coined by Funk[7] as the result of an attempt to isolate from food the substance, of as yet unknown nature, that prevented the disease, beri-beri. When beri-beri thus came to be recognized as a " vitamine " deficiency disease, the hypothesis was advanced that various other " vitamine " deficiency diseases occur. At about the same time, laboratory studies in nutrition showed that natural foods contain chemical substances necessary for normal nutrition, but not classified under any of the ordinary groups of nutrients, *i.e.*, protein, carbohydrate, fat, and mineral salts. Of especial importance was the discovery that these substances were incapable of chemical determination by the methods applicable to the nutrients mentioned. Particularly astonishing was the fact that the effect exerted by these accessory food ingredients was far greater than that shown by corresponding amounts of known nutrients and apparently quite out of proportion to the amounts evidently present in the food. The workers in the field of " vitamine " deficiency diseases and those studying the strictly nutrition problems soon came to recognize that these new substances required for normal nutrition were apparently identical with the "vitamines," a lack of which was primarily concerned with the etiology of the deficiency diseases. It was in this way that these two fields of investigation came together and contributed to the discovery of and the extension of our knowledge of vitamins. Advances in our knowledge of the vitamins at the present time are due to the same methods of attack, *i.e.*, through the study of particular diseases and by laboratory studies in nutrition. Although the existence of vitamins seems to be well established through these methods of study, it must not be overlooked that it remains for chemistry to establish their existence through isolation, identification, and synthesis.

There have been various reports of the isolation of one or more of the vitamins. It will not be possible to accept these findings until they are confirmed by other workers. Probably the most definite information we have at the present time regarding the nature of a vitamin

[7] Funk, C., The Etiology of the Deficiency Diseases, *J. State Med.*, 20 : 341–368 (1912).

is disclosed by the relation of vitamin D to ergosterol, discussed on pages 651–654. Chemical proof of the existence of the other vitamins is confined to, (a) their separation from natural foods by chemical means and their concentration in the extracts thus obtained, by means of adsorption, or distillation, or simple evaporation; (b) the establishing of certain important chemical properties; and (c) the determination of the character of the chemical reactions which destroy them. The last mentioned field of study is not only of great importance in establishing the chemical existence of vitamins but, because of their great importance in nutrition, it has given data of great practical value to those engaged in various food manufacturing processes.

As already mentioned, one of the fascinating features of vitamin nutrition is the tremendous disproportion between their importance and the amounts normally consumed. Because they contribute no significant material substance or energy to the body, but act rather as catalysts of life processes, it is necessary to place vitamins in an entirely different category from the classification of the better known foodstuffs, such as proteins, fats, carbohydrates, and mineral salts.

Vitamins are primarily plant products. There appear to be certain exceptions to this rule, the primary exception being that ergosterol or some like sterol, whether in plant or animal tissue, is capable of being transformed into vitamin D under the influence of the proper light rays. Another apparent exception is the fact that some animals appear to be able to synthesize vitamin C. It has at any rate been established for the rat,[8] the calf,[9] and the chicken,[10] that the liver contains an abundance of vitamin C when the animals have been fed for long periods or reared on diets which lack this factor. These findings show that these species are not dependent on their food for vitamin C, and have been interpreted as showing vitamin C synthesis in the animal itself. The lack of dependence of growing cattle on their rations for vitamins B and G[11] has been explained by the finding[12] that these factors are synthe-

[8] Parsons, H. T., The Antiscorbutic Content of Certain Body Tissues of the Rat. The Persistence of the Antiscorbutic Substance in the Liver of the Rat after Long Intervals on a Scorbutic Diet, *J. Biol. Chem.*, 44 : 587–602 (1920).

[9] Thurston, M. L., The Role of the Antiscorbutic Vitamin in the Nutrition of Calves, Thesis for the Ph.D. degree, filed in the Library, University of Minnesota (1928).

[10] Carrick, C.W., and Hauge, S. M., Presence of the Antiscorbutic Substance in the Livers of Chickens Fed on Scorbutic Diets, *J. Biol. Chem.*, 63 : 115–122 (1925).

[11] Bechdel, S. I., Eckles, C. H., and Palmer, L. S., The Vitamin B Requirement of the Calf, *J. Dairy Sci.*, 9 : 409–438 (1926).

[12] Bechdel, S. I., Honeywell, H. E., Dutcher, R. A., and Knutsen, M. H., Synthesis of Vitamin B in the Rumen of the Cow, *J. Biol. Chem.*, 80 : 231–238 (1928).

sized by the bacteria inhabiting the rumen of this species. The human organism, however, apparently requires all the vitamins at present recognized, although this has not been definitely established for vitamin E. In addition, man is dependent on food for his vitamins, with the exception of vitamin D which is formed in the epidermis after exposure to ultraviolet rays.

An important nutrition fact in connection with vitamins is the ability of animals to store these factors in the organs of the body, particularly in the liver. This is particularly true of vitamin A, and for fish, of vitamin D. In spite of the fact that the other vitamins are not stored in any great quantities, there is little evidence to indicate that excessive doses of the vitamins exert deleterious effects. A possible exception is that of proprietary preparations of irradiated ergosterol.

The phases of vitamin knowledge which are probably of most interest from the biochemical standpoint have to do with the effects of the presence or absence of vitamins on animal life, their origin and distribution in nature, and their chemical properties. The subsequent paragraphs will present briefly such facts, bearing on these phases of the vitamins, as appear to be of especial importance.

Fat-soluble Vitamins.—Vitamin A.—Vitamin A was discovered almost simultaneously by McCollum and Davis[13] and by Osborne and Mendel.[14] The discovery was based on the observations which have been repeatedly confirmed, that certain fats and oils possess a remarkable power of stimulating the growth of rats, which stimulatory effect is absent in whole or in part from other fats and which is not explainable on the basis of the glycerides contained in these fats. Several years later Drummond[15] studied in detail the known components of growth-promoting fats and was unable to identify the vitamin with glycerol, saturated or unsaturated fatty acids, cholesterol, lecithin, and other phospholipides.

Vitamin A is classified among the growth-promoting factors. It is not clear just how this effect is brought about. There is no evidence as yet that this or any other vitamin actually accelerates the metabolic processes or modifies their efficiency. The probabilities are that the growth-promoting effect of vitamin A is exerted indirectly through its apparent ability to maintain the integrity of the epithelial tissues of the

[13] McCollum, E. V., and Davis, M., The Necessity of Certain Lipins in the Diet During Growth, *J. Biol. Chem.*, 15 : 167–175 (1913).

[14] Osborne, T. B., and Mendel, L. B., The Relation of Growth to the Chemical Constituents of the Diet, *J. Biol. Chem.*, 15 : 311–326 (1913).

[15] Drummond, J. C., Researches on the Fat-Soluble Accessory Substance. I. Observations upon Its Nature and Properties, *Biochem. J.*, 13 : 81–94 (1919).

animal body. This is supported by the fact that the primary effect of a deficiency of vitamin A is a keratinization of the epithelial tissues. There is no specific disease which is characteristic of vitamin A deficiency. There is no longer any foundation for the general assumption that xerophthalmia is a specific disease due to lack of vitamin A. As a matter of fact, the diseased condition of the cornea, which is called xerophthalmia, is the result of bacterial invasion of the keratinized tissue.[16-18] Mori found the keratinized condition of the epithelium to extend to the salivary gland, mouth, larynx, trachea, bronchi, and lungs. These findings were verified by Wolbach and Howe,[19] who found in addition that the keratinization extended throughout the alimentary tract, the genital-urinary tract, and various ductless glands. These findings show why vitamin A deficiency in animals lowers their resistance to respiratory diseases and also explain the relation of the vitamin to reproduction. These facts emphasize the importance of this vitamin for human nutrition, particularly since it does not have a wide distribution in nature among the foods commonly employed by man.

Vitamin A is synthesized by plants only, particularly in green leaves where it is found in highest concentration in the plant kingdom. This vitamin occurs abundantly also in green and yellow seeds and in various roots and fruits which also contain the yellow carotinoid pigments.[20-25]

[16] Mori, S., Primary Changes in Eyes of Rats Which Result from Deficiency of Fat-Soluble A in Diet, *J. Am. Med. Assoc.*, 79 : 197–200 (1922).

[17] Mori, S., The Changes in the Para-Ocular Glands Which Follow the Administration of Diets Low in Fat-Soluble A; With Notes of the Effect of the Same Diets on the Salivary Glands and the Mucosa of the Larynx and Trachea, *Bull. Johns Hopkins Hosp.*, 33 : 357–359 (1922).

[18] Mori, S., The Pathological Anatomy of Ophthalmia Produced by Diets Containing Fat-Soluble A, but Unfavorable Contents of Certain Inorganic Elements, *Am. J. Hyg.*, 3 : 99–102 (1923).

[19] Wolbach, S. B., and Howe, P. R., Tissue Changes Following Deprivation of Fat-Soluble A Vitamin, *J. Exp. Med.*, 42 : 753–777 (1925).

[20] Steenbock, H., Boutwell, P. W., and Kent, H. E., Fat-Soluble Vitamine, I., *J. Biol. Chem.*, 35 : 517–526 (1918).

[21] Steenbock, H., and Gross, E. G., Fat-Soluble Vitamine. II. The Fat-Soluble Vitamine Content of Roots, Together with Some Observations on Their Water-Soluble Vitamine Content, *J. Biol. Chem.*, 40 : 501–531 (1919).

[22] Steenbock, H., and Boutwell, P. W., Fat-Soluble Vitamine. III. The Comparative Nutritive Value of White and Yellow Maizes, *J. Biol. Chem.*, 41 : 81–96 (1920).

[23] Steenbock, H., and Gross, E. G., Fat-Soluble Vitamine. IV. The Fat-Soluble Vitamine Content of Green Plant Tissues Together with Some Observations on Their Water-Soluble Vitamine Content, *J. Biol. Chem.*, 41 : 149–162 (1920).

[24] Steenbock, H., and Boutwell, P. W., Fat-Soluble Vitamine. VI. The Extractability of the Fat-Soluble Vitamine from Carrots, Alfalfa, and Yellow Corn by Fat Solvents, *J. Biol. Chem.*, 42 : 131–152 (1920).

It is not known whether the vitamin is synthesized in these tissues or transported there after synthesis in the leaves. A physiological relationship between vitamin A and the carotinoids in plants has not yet been discovered. The genetic factors for carotinoid pigmentation and vitamin A occurrence in yellow maize cannot, however, be separated by crossing white and yellow varieties.[26]

Among the foods of animal origin which are usually reliable sources of vitamin A may be mentioned whole milk, butter, cheese, egg yolk, and the glandular organisms of animals, particularly the liver. The presence of vitamin A in these foods, however, depends upon the presence of the vitamin in the food of the animals which produce these products.[27] There is no evidence whatever that animals are able to synthesize vitamin A. Properly prepared cod liver oil is an especially rich source of vitamin A, as is the oil from the liver of many other fish. This fact is of considerable importance when therapeutic doses of the vitamin are necessary. There is good evidence to indicate that these high concentrations of vitamin A in the liver of fish represent the effect of diets exceptionally rich in the vitamin during the fattening season. A similar relationship exists between the diet of cows and hens and the vitamin A content of milk and eggs. The highest concentrations of vitamin A in these foods occur during the season when an abundance of fresh green forage is available. Fortunately the lack of green forage for the winter feeding of dairy cattle and laying fowls may be overcome in large part through the use of properly cured, leafy forage, yellow seeds, such as yellow corn, and yellow roots. These facts are valuable to the producers of eggs and milk, in helping them control the vitamin A content of their respective products.

The most recent experiments on vitamin A synthesis in plants[28-30] show that no vitamin A synthesis occurs in the etiolated shoots of wheat germinated in the dark for two to three days. When growth is allowed to

[25] Steenbock, H., Sell, M. T., and Boutwell, P. W., Fat-Soluble Vitamine. VIII. The Fat-Soluble Vitamine Content of Peas in Relation to Their Pigmentation, *J. Biol. Chem.*, 47 : 303–308 (1921).

[26] Hauge, S. M., and Trost, J. F., An Inheritance Study of the Distribution of Vitamin A in Maize, *J. Biol. Chem.*, 80 : 107–114 (1928).

[27] Kennedy, C., and Dutcher, R. A., Vitamine Studies. IX. The Influence of the Diet of the Cow upon the Quantity of Vitamines A and B in the Milk, *J. Biol. Chem.*, 50 : 339–359 (1922).

[28] Coward, K. H., The Influence of Light and Heat on the Formation of Vitamin A in Plant Tissues, *J. Biol. Chem.*, 72 : 781–799 (1927).

[29] Moore, T., Vitamin A Formation in the Etiolated Wheat Shoot, *Biochem. J.*, 21 : 870–874 (1927).

[30] Heller, V. G., Vitamin Synthesis in Plants as Affected by Light Source, *J. Biol. Chem.*, 76 : 499-511 (1928).

continue in the dark for ten to sixteen days, however, very appreciable amounts of vitamin A are present. It appears, therefore, that vitamin A is synthesized to some extent in the later stages of germination without the photochemical acceleration of light. Light, however, greatly accelerates vitamin A synthesis, but apparently does not control the ultimate amounts in the plant. The shorter light rays are particularly effective but are not exclusively concerned in the process. Apparently vitamin A synthesis goes hand in hand with chlorophyll formation, at least in green plants. Widmark[31] believes that plants which lose chlorophyll, likewise lose their ability to synthesize vitamin A.

The chemical nature of vitamin A remains to be discovered. It is not improbable that it, like vitamins D and E, is related to the sterols. At any rate, the most concentrated forms of this vitamin so far prepared by Takahashi,[32-35] and by Drummond, Channon, and Coward[36] appear to be practically exclusively composed of sterols. Takahashi, who first claimed the isolation of vitamin A, called his product, " biosterin," and ascribed to it the formula, $C_{27}H_{44}(OH)_2$. It is unfortunately impossible to accept Takahashi's claims in view of the failure of Drummond, Channon, and Coward to confirm the essential features of his work. The earlier view of Steenbock[37] that vitamin A is either carotin or always associated with carotin was not confirmed by later investigation (cf. Drummond,[38] Stephenson,[39] Palmer and Kennedy,[40] Steen-

[31] Widmark, E., Der Zusammenhang zwischen der Bildung des A-Vitamins und den Farbstoffen der Pflanzen, *Skand. Arch. Physiol.*, 45 : 7–11 (1924).

[32] Takahashi, K., Nutritive Values of Lipoids. IV. Separation and Identification of the Active Principle (Vitamin A) of Cod-Liver Oil, *Proc. Japan. Chem. Soc.; J. Chem. Soc., Japan*, 43 : 828–830 (1922), (in Japanese) *cf. Chem. Abs.*, 17 : 1984 (1923).

[33] Takahashi, K., and Kawakami, K., Chemistry of Vitamin A. I. Separation of the Effective Constituent of the Liver Oil and Its Properties, *J. Chem. Soc., Japan*, 44 : 590–605 (1923), (in Japanese) *cf. Chem. Abs.*, 20 : 1653 (1926).

[34] Takahashi, K., Nakamiya, Z., Kawakami, K., and Kitasato, T., On the Physical and Chemical Properties of Biosterin (A Name Given to Fat-Soluble A) and on Its Physiological Significance, *Sci. Papers Inst. Phys. Chem. Res.*, 3 : 81–145 (1925).

[35] Takahashi, K., and Nakamiya, J., Physiological Significance of Biosterin (So-Called Vitamin A), *Japan Medical World*, 5 : 2–9 (1925).

[36] Drummond, J. C., Channon, H. J., and Coward, K. H., Studies on the Chemical Nature of Vitamin A, *Biochem. J.*, 19 : 1047–1067 (1925).

[37] Steenbock, H., White Corn Versus Yellow Corn and a Probable Relation between the Fat-Soluble Vitamine and Yellow Plant Pigments, *Science*, 50 : 352–353 (1919).

[38] Drummond, J. C., Researches on the Fat-Soluble Accessory Substance. I. Observations upon Its Nature and Properties, *Biochem. J.*, 13 : 81–94 (1919).

[39] Stephenson, M., A Note on the Differentiation of the Yellow Plant Pigments from the Fat-Soluble Vitamine, *Biochem. J.*, 14 : 715–720 (1920).

[40] Palmer, L. S., and Kennedy, C., The Relation of Plant Carotinoids to Growth and Reproduction of Albino Rats, *J. Biol. Chem.*, 46 : 559–577 (1921).

bock, Sell, and Buell,[41] Coward,[42] and Drummond, Channon, and Coward[43]).

Some of the important chemical properties of vitamin A, established by the work of many investigators, are as follows:

The vitamin is non-saponifiable and may be recovered in the non-saponifiable fraction of fats and oils which contain it. The same is true of extracts of plant tissues prepared for the purpose of concentrating the vitamin.

Vitamin A is readily oxidized and loses its potency during the process. This fact explains why vitamin A is heat-stable under certain conditions and very unstable to heating under other conditions. So readily is vitamin A oxidized that complete recovery in the non-saponifiable fraction of fats and oils is assured only when saponification and extraction of the soap are carried out in an inert atmosphere.

The heat stability of vitamin A is demonstrated by the fact that it is volatile in super-heated steam at 115–125° C. and can be concentrated by fractional distillation, the vitamin coming over at 250° C. (3–4 mm.) or at 180–220° C. (2–3 mm.) (cf. Drummond and Coward[44]). The distillation in steam must be carried out in a nitrogen atmosphere and is very tedious. However, the crude vitamin thus obtained is concentrated 250 to 300 times in comparison with the original fat. According to Drummond, Channon, and Coward, vitamin A comes over in greatest quantity in a pale yellow oil.

Vitamin A appears to have associated with it a substance which gives a very beautiful color reaction. While there is as yet no quantitative evidence that it is specific for vitamin A, the test is undoubtedly the most important of the color tests for the vitamins so far discovered. Drummond and his coworkers were able to use the test to follow the fractionations of their vitamin A concentrates by distillation. The test is particularly applicable to qualitative detection of vitamin A in fresh liver oil, particularly cod-liver oil, and has been suggested[45] as a quantitative

[41] Steenbock, H., Sell, M. T., and Buell, M. V., Fat-Soluble Vitamine. VII. The Fat-Soluble Vitamine and Yellow Pigmentation in Animal Fats with Some Observations on Its Stability to Saponification, *J. Biol. Chem.*, 47 : 89–109 (1921).

[42] Coward, K. H., The Association of Vitamin A with the Lipochromes of Plant Tissues, *Biochem. J.*, 17 : 145–156 (1923).

[43] Drummond, J. C., Channon, H. J., and Coward, K. H., Studies on the Chemical Nature of Vitamin A, *Biochem. J.*, 19 : 1047–1067 (1925).

[44] Drummond, J. C., and Coward, K. H., Chemical Studies of the Vitamin A in Cod-Liver Oil, *J. Soc. Chem. Ind.*, 43 : 544–545 (1924).

[45] Rosenheim, O., and Schuster, E., A New Colorimeter Based on the Lovibond Colour System, and Its Application to the Testing of Cod-Liver Oil, and Other Purposes, *Biochem. J.*, 21 : 1329–1334 (1927).

measure of the vitamin. It is a modification of the discovery of Hager,[46] that a drop of concentrated sulfuric acid added to a drop of cod liver oil dissolved in a few drops of petroleum ether, chloroform, or carbon tetrachloride gives a deep blue color. Drummond and Watson[47] discovered that the intensity of this reaction in cod-liver oil paralleled the growth-promoting power of the oil and that a destruction of the biological property was accompanied by a destruction of the color reaction. These investigators found that liver oils from man, horse, ox, pig, cat, monkey, rabbit, guinea pig, chicken, duck, pigeon, rat, mouse, frog, shark, cod, haddock, ling, coal fish, dog fish, sprat, and skate give the reaction. Many modifications of this reaction have been proposed to increase its sensitiveness. Perhaps the most commonly employed modifications are those of Rosenheim and Drummond,[48] who employed arsenic trichloride as the reagent in place of sulfuric acid, or of Carr and Price,[49] who employed antimony trichloride. The arsenic trichloride is said to give the reaction with cholesterol-free, non-saponifiable matter from cod-liver oil in dilution of 1 : 2,000,000. The antimony trichloride test, which is much more convenient to use because of the lack of objectionable fumes, may be carried out as follows (cf. Willimott, Moore, and Wokes[50]). Antimony trichloride crystals washed with anhydrous chloroform are dissolved in the chloroform to form a 30 per cent solution (weight in volume). A 20 per cent (by volume) solution in chloroform of the oil to be tested is prepared, and 0.2 cc. of the oil solution is added to 2 cc. of reagent.

Vitamin D.—Hopkins[51] apparently first recognized the possibility that some "accessory" dietary factor is concerned in the etiology of rickets. Funk[52] later classified rickets as a hypothetical avitaminosis. Mellanby[53] clearly demonstrated for the first time that rickets may be

[46] Hager, H., Zur Prüfung des Leberthrans, *Pharm. Zentralhalle*, 26 : 13–16 (1885).

[47] Drummond, J. C., and Watson, A. F., The Sulphuric Acid Reaction for Liver Oils, *Analyst*, 47 : 341–349 (1922).

[48] Rosenheim, O., and Drummond, J. C., A Delicate Colour Reaction for the Presence of Vitamin A, *Biochem. J.*, 19 : 753–756 (1925).

[49] Carr, F. H., and Price, E. A., Colour Reactions Attributed to Vitamin A, *Biochem. J.*, 20 : 497–501 (1926).

[50] Willimott, S. G., Moore, T., and Wokes, F., Effects of Various Agents on Color Tests for Vitamin A, *Biochem. J.*, 20 : 1292–1298 (1926).

[51] Hopkins, F. G., The Analyst and the Medical Man, *Analyst*, 31 : 385–404 (1906). (Cf. p. 395.)

[52] Funk, C., Die Vitamine, ihre Bedeutung für Physiologie und Pathologie mit besonderer Berucksichtigung der Avitaminosen (Beri-beri, Skorbut, Pellagra, Rachitis), J. F. Bergmann, Wiesbaden (1914).

[53] Mellanby, E., An Experimental Investigation of Rickets, *Lancet*, 196 : 407-412 (1919).

brought about by a deficiency of fat-soluble vitamin in the diet. Since vitamin A was the only fat-soluble vitamin recognized at that time, it is not surprising that vitamin A was believed to be the factor concerned with the prevention of a rachitic condition. It remained for McCollum[54] and his coworkers to provide the first experimental demonstration of the existence of the distinct fat-soluble vitamin related to the etiology of rickets. This vitamin is now known as vitamin D.

It does not seem desirable to discuss at length the pathology of rickets. It should be pointed out, however, that this disease is not entirely a vitamin-deficiency disease. Vitamin D does not have the same degree of responsibility for the prevention of rickets as do the other vitamins for the specific diseases with which they are concerned. The relative proportions of calcium and phosphorus in a diet are also important factors in the etiology of rickets. The function of vitamin D seems to be to maintain the proper concentration of calcium and phosphorus in the blood regardless of any disproportion between these elements in the food. Vitamin D, therefore, exerts its most spectacular effect in the face of abnormal mineral composition of the diet. When the latter is normal, particularly with reference to calcium and phosphorus, the need for vitamin D is apparently at a minimum. Of the two elements, calcium and phosphorus, whose metabolism is regulated by vitamin D, phosphorus appears to be the more easily influenced. The manner in which vitamin D exerts its regulatory influence is not yet entirely clear. The evidence at present indicates that it is in connection with absorption and excretion along the intestinal tract that vitamin D performs its remarkable functions. In fact, the biochemistry of the processes involved is as yet obscure.

The relation of vitamin D to rickets has led to the general assumption that this vitamin is concerned solely with the metabolism of bone. It should be borne in mind, however, that the first effect of vitamin D deficiency is probably a decrease in the concentration of phosphorus in the blood. Inasmuch as phosphorus is concerned in the metabolism of nearly all body tissues, it is to be expected that rickets will be accompanied by abnormal metabolism in other tissues than the bones and that vitamin D will exert important effects other than in the normal development of bone. These facts afford an explanation for the general muscular weakness and instability of the nervous system which accompany rickets. Phosphorus is an important element in the normal metabolism of these tissues. It is also to be expected that vitamin D may be

[54] McCollum, E. V., Simmonds, N., Becker, J. E., and Shipley, F. G., An Experimental Demonstration of the Existence of a Vitamin Which Promotes Calcium Deposition, *Bull. Johns Hopkins Hosp.*, 33 : 229–230 (1922).

classed among the growth-promoting vitamins, a fact first clearly pointed out by Steenbock and Nelson.[55]

The origin of vitamin D appears to be explained satisfactorily through its relation to ergosterol, discussed in Chapter XXXI. The fact that ergosterol may be transformed into vitamin D by ultraviolet rays shorter than 3100 Å indicates that the supply of this factor to meet the needs of man and animals is limited only by the distribution of ergosterol and an available source of the necessary light rays. So far as the matter has been investigated, it appears that all plants and animals contain the provitamin, inasmuch as the exposure to the activating light rays has generated vitamin D in all plants so far investigated and in animal tissues, as well as in living animals themselves. In view of this fact it seems surprising that vitamin D has such a limited distribution in nature, particularly among plants grown with exposure to sunlight. One explanation that has been offered for this fact is that our methods of detection of vitamin D are not sensitive enough to reveal the presence of this factor in such material. Another plausible explanation is afforded by the observation that vitamin D may be destroyed by ultraviolet rays as well as generated by them. The first explanation is weakened by the fact that as little as 0.00002 mg. (probably by no means the minimum dose) per day of irradiated ergosterol is effective in curing experimental rickets in a rat. If the second explanation seems more acceptable, it is evident that very little of the provitamin has been activated and destroyed during the natural exposure to sunlight. All plants and plant products so far investigated are still capable of being strongly activated by an artificial source of ultraviolet rays. Whatever the explanation may be, it is obvious that man has found a way of improving on nature in the production of vitamin D.

Very little can be said as yet regarding the chemical properties of vitamin D, inasmuch as all efforts so far have failed to isolate the active substance formed from ergosterol. The vitamin strongly resists crystallization. Inasmuch as ergosterol apparently is not quantitatively changed to vitamin D, it is obvious that the chemical properties of activated ergosterol will not be solely those of the vitamin. It is known that many of the original chemical properties of ergosterol are changed by irradiation, but it is difficult to interpret these results in terms of pure vitamin.

Vitamin E.—Vitamin E was discovered by Evans and Bishop[56, 57]

[55] Steenbock, H., and Nelson, E. M., Fat-Soluble Vitamine. XIII. Light in Its Relation to Ophthalmia and Growth, *J. Biol. Chem.*, 56 : 355–373 (1923).

[56] Evans, H. M., and Bishop, K. S., On the Existence of a Hitherto Unrecognized Dietary Factor Essential for Reproduction, *Science*, 56 : 650–651 (1922).

in a study of the oestrous cycle of female rats fed for relatively long periods upon synthetic diets with an ample provision for all of the previously known vitamins. They found that reproductive failure occurred which was very characteristic in its nature. Although ovulation took place, and the foeti were implanted and began to develop normally, the placental function failed before parturition, with the accompanying death and resorption of the implanted foeti. Reproduction in the sense of failure for young to appear may occur as a result of many other dietary deficiencies. It is to be emphasized, therefore, that vitamin E deficiency can be detected only through the special histological technic developed by Evans and Bishop. It should also be emphasized that up to this time the need for this vitamin by animals has been demonstrated only for the rat.

The physiological effects of vitamin E deficiency are manifested in other ways. A type of male sterility[58-63] also develops, although much more slowly than the reproductive failures in the female. The effects on the male may be permanent, but this is not likely to be the case for female animals. Evans and Burr[64] have studied a form of paralysis in suckling young rats nursed by mothers on certain diets. This condition can be cured by changing the diet to natural food, or prevented by foods rich in vitamin E or by vitamin E rich concentrate prepared from wheat germ oil. It would appear, therefore, that vitamin E performs some necessary function during the early stages of growth. Much remains to be learned, however, regarding this newly discovered function of vitamin E.

Vitamin E appears to be widely distributed in natural foods.

[57] Evans, H. M., and Bishop, K. S., On the Relations between Fertility and Nutrition, I–IV, *J. Metabolic Research*, 1 : 319–333; 335–356 (1922); 3 : 201–231; 233–316 (1923).

[58] Mattill, H. A., Carman, J. S., and Clayton, M. M., The Nutritive Properties of Milk. III. The Effectiveness of the X Substance in Preventing Sterility in Rats on Milk Rations High in Fat, *J. Biol. Chem.*, 61 : 729–740 (1924).

[59] Mason, K. E., A Histological Study of Sterility in the Albino Rat Due to a Dietary Deficiency, *Proc. Nat. Acad. Sci.*, 11 : 377–382 (1925).

[60] Evans, H. M., Invariable Occurrence of Male Sterility with Dietaries Lacking Fat Soluble Vitamine E, *Proc. Nat. Acad. Sci.*, 11 : 373–377 (1925).

[61] Mason, K. E., Testicular Degeneration in Albino Rats Fed a Purified Food Ration, *J. Exp. Zool.*, 45 : 159–229 (1926).

[62] Mattill, H. A., The Relation of Vitamins B and E to Fertility in the Male Rat, *Am. J. Physiol.*, 79 : 305–315 (1927).

[63] Evans, H. M., and Burr, G. O., The Antisterility Vitamine Fat Soluble E, Memoirs of the University of California, Vol. 8 (1927).

[64] Evans, H. M., and Burr, G. O., Development of Paralysis in the Suckling Young of Mothers Deprived of Vitamin E, *J. Biol. Chem.*, 76 : 273–297 (1928).

Although fat-soluble, it does not seem to be concentrated exclusively in the oil-bearing portions of plants. Various animal tissues contain this factor, though in less highly concentrated form, *e.g.*, adipose tissue, muscle meat, visceral organs, and milk, particularly milk fat.[65] Egg yolk appears to lack vitamin E. Among the foods of plant origin which have been found to contain vitamin E in sufficient concentration to be effective in preventing experimental reproductive failures with rats, may be mentioned lettuce or alfalfa leaves, either fresh or dry, various seeds including the cereals, alfalfa seed, and lettuce seed, and raw peanuts. Among the fruits, bananas, tomatoes, and oranges appear to contain vitamin E. Many vegetable oils have been found to contain this vitamin, but few in a very high concentration. The most potent oil so far studied seems to be the oil which may be extracted from wheat germ. Oil from the other cereals, such as rice, barley, and oats, is not as effective as wheat germ oil in the cure or prevention of vitamin E deficiency.

Most of the chemical properties so far ascertained for vitamin E are the result of the studies of Evans and Burr.[66, 67] The most concentrated preparations so far obtained obviously contain vitamin E merely as an impurity. The effective dose of this concentrate is about 5 milligrams. While one dose is sufficient to carry a female rat through one or more gestation periods, it is evident that the concentrate contains vitamin E only as a minor fraction, if the efficiency of vitamin E is at all comparable quantitatively to that of vitamin D.

Vitamin E appears to be remarkably stable to heat, light, air, and many ordinary chemical reactions. It may be distilled in steam or *in vacuo*. It is not readily oxidized by chemical means, but apparently is either destroyed during the chemical oxidation of fat, or the oxidation products of fat prevent the vitamin from exerting its normal biological effect.[68-70] Vitamin E is not destroyed during the hydrogenation of fat which contains it, as evidenced by the fact that commercially

[65] Evans, H. M., and Burr, G. O., The Antisterility Vitamine Fat Soluble E, Memoirs of the University of California, Vol. 8 (1927).

[66] Evans, H. M., and Burr, G. O., The Antisterility Vitamine Fat Soluble E, *Proc. Nat. Acad. Sci.*, 11 : 334–341 (1925).

[67] Evans, H. M., and Burr, G. O., The Antisterility Vitamine Fat Soluble E, Memoirs of the University of California, Vol. 8 (1927).

[68] Evans, H. M., and Burr, G. O., Vitamin E. The Ineffectiveness of Curative Dosage When Mixed with Diets Containing High Proportions of Certain Fats, *J. Am. Med. Assoc.*, 88 : 1462–1465 (1927).

[69] Mattill, H. A., The Oxidative Destruction of Vitamins A and E, and the Protective Action of Certain Vegetable Oils, *J. Am. Med. Assoc.*, 89 : 1505–1508 (1927).

[70] Evans, H. M., and Burr, G. O., Vitamin E. II. The Destructive Effect of Certain Fats and Fractions Thereof on the Antisterility Vitamin in Wheat Germ and in Wheat Germ Oil, *J. Am. Med. Assoc.*, 89 : 1587–1590 (1927).

hydrogenated cottonseed oil still contains the vitamin. Vitamin E may be concentrated through saponification of oils containing it and recovery of the non-saponifiable matter by the usual methods. Prolonged hot saponification partially destroys it, making it necessary to carry out this reaction at moderate temperatures. Although the most concentrated preparations have properties like the sterols, vitamin E apparently does not belong to one of the known members of this group. Unlike ergosterol it is not precipitated by digitonin. Cold pentane has proved to be an excellent solvent for vitamin E, inasmuch as many of the substances which accompany vitamin E in the non-saponifiable fractions of oils are not soluble in this solvent.

WATER-SOLUBLE VITAMINS.—VITAMIN B.—This term is reserved for the vitamin which is frequently referred to as the antineuritic or antiberi-beri substance. So many workers contributed to the early history of this subject that it is difficult now to ascribe to any one worker the full credit for the discovery of an antineuritic vitamin. The experiments of Eijkman,[71,72] who first produced experimental beri-beri, and cured and prevented it by use of rice polishings, may be now interpreted as showing the existence of such a vitamin. Eijkman did not himself so interpret it until later.[73]

Attempts to isolate an antiberi-beri substance perhaps began with Hulshoff-Pol.[74] The search for the antiberi-beri substance was prosecuted intensively during the years 1910–1912, [75-85] and culminated

[71] Eijkman, C., Polyneuritis bij Hoenderen, *Geneesk, Tijdschr. v. Ned. Ind.*, 30 : 295–334 (1890).

[72] Eijkman, C., Polyneuritis bij Hoenders. Nieuwe Bijdrage tot de Aetiologie der Ziekte, *Geneesk. Tijdschr. v. Ned. Ind.*, 36 : 214–269 (1896).

[73] Eijkman, C., Über Ernährungspolyneuritis, *Arch. Hyg.*, 58 : 150–170 (1906).

[74] Hulshoff-Pol, D. J., X-zuur; het tegen beri-beri verkzame bestanddeel uit de Katjang-hidjoe, *Geneesk. Tijdschr. v. Ned. Ind.*, 47 : 688–702 (1907).

[75] Schaumann, H., Beriberi und Nucleinphosphorsäure in der Nahrung, *Arch. Schiffs-Tropen-Hyg. Beiheft*, 12 : 37–57 (1908).

[76] Schaumann, H., Weitere Beiträge zur Ätiologie der Beriberi, *Arch. Schiffs-Tropen-Hyg. Beiheft*, 13 : 82–90 (1909).

[77] Schaumann, H., Die Ätiologie der Beriberi unter Berücksichtigung des gesamten Phosphorstoffwechsels, *Arch. Schiffs-Tropen-Hyg. Beiheft*, 14 : 325–709 (1910). (Includes a bibliography of 294 citations.)

[78] Aron, H., Phosphorus Starvation with Special Reference to Beri-Beri. I., *Philippine J. Sci.*, Sect. B, 5 : 81–97 (1910).

[79] Aron, H., and Hocson, F., Phosphorus Starvation with Special Reference to Beri-Beri. II., *Philippine J. Sci.*, Sect. B., 5 : 98–122 (1910).

[80] Fraser, H., and Stanton, A. T., The Etiology of Beri-Beri, *Lancet*, 179 : 1755–1757 (1910).

[81] Chamberlain, W. P., and Vedder, E. B., A Contribution to the Etiology of Beriberi, *Philippine J. Sci.*, Sect. B, 6 : 251–258 (1911).

with the coining of the term, " vitamine," by Funk[86] for the substance which he had isolated and which be believed to be the specific antineuritic substance. Funk's " vitamine " later proved to be largely nicotinic acid which does not have antineuritic properties. Although many workers have made highly concentrated preparations of the antineuritic vitamin, no one has as yet been successful in isolating it and determining its exact chemical nature. The recent claims of Jansen and Donath[87] for the isolation of the factor remain to be verified, although the antineuritic action of their preparation cannot be questioned, inasmuch as doses of 0.012 mg. daily were sufficient to protect pigeons from experimental polyneuritis. A number of other investigators have also obtained highly concentrated antineuritic preparations.[88-91]

Although the exact chemical nature of vitamin B is still undetermined, it has long been known[92] that it is a nitrogenous base. Funk[93] showed that it appears for the largest part in the arginine fraction when submitted to the Kossel separation of the hexone bases. An especially interesting series of investigations regarding the probable nature of vitamin B is that of Williams[94-97] who prepared and tested many

[82] Chamberlain, W. P., and Vedder, E. B., A Second Contribution to the Etiology of Beriberi, *Philippine J. Sci.*, Sect. B, 6 : 395–404 (1911).

[83] Schaumann, H., Further Contribution to the Etiology of Beriberi, *Trans. Soc. Trop. Med. Hyg.*, 5 : 59–75 (1911).

[84] Chamberlain, W. P., Vedder, E. B., and Williams, R. R., A Third Contribution to the Etiology of Beriberi, *Philippine J. Sci.*, Sect. B., 7 : 39–52 (1912).

[85] Suzuki, U., Shimamura, T., and Odake, S., Über Oryzanin, ein Bestandteil der Reiskleie und seine physiologische Bedeutung, *Biochem. Z.*, 43 : 89–153 (1912).

[86] Funk, C., The Etiology of the Deficiency Diseases, *J. State Med.*, 20 : 341–368 (1912).

[87] Jansen, B. C. P., and Donath, W. F., Isolation of Anti beriberi-vitamin, *Mededeel. Dienst Volksgezondheid Nederland-Indië*, Pt. I : 186–199 (1927).

[88] Seidell, A., Further Experiments on the Isolation of the Antineuritic Vitamin, *J. Am. Chem. Soc.*, 44 : 2042–2051 (1922).

[89] Seidell, A., The Chemistry of Vitamins, *Science*, 60 : 439–447 (1924).

[90] Levene, P. A., The Concentration of Vitamin B, IV. On the Concentration and the Separation of the Two Components of Vitamin B, *J. Biol. Chem.*, 79 : 465–470 (1928).

[91] Kinnersley, H. W., and Peters, R. A., Antineuritic Yeast Concentrates. IV. The Further Purification of Yeast Vitamin B_1 (Curative), *Biochem. J.*, 22 : 419–433 (1928).

[92] Chamberlain, W. P., Vedder, E. B., and Williams, R. R., A Third Contribution to the Etiology of Beri-beri, *Philippine J. Sci.*, Sect. B., 7 : 39–52 (1912).

[93] Funk, C., On the Chemical Nature of the Substance Which Cures Polyneuritis in Birds Induced by a Diet of Polished Rice, *J. Physiol.*, 43 : 395–400 (1911).

[94] Williams, R. R., The Chemical Nature of the " Vitamines." I. Antineuritic Properties of the Hydroxypyridines, *J. Biol. Chem.*, 25 : 437–445 (1916).

pyridine compounds. His extensive studies led him to the belief that the antineuritic vitamin eventually will be found to be a cyclic nitrogen compound with an oxygen substitution on the ring which is capable of existence in a betaine configuration. Certain of Williams' experiments were not confirmed by Harden and Zilva,[98] but their work is not very convincing, particularly in view of the apparent confirmation of Williams' investigations by Hofmeister.[99]

Our knowledge of the pathological effects of vitamin B deficiency is complicated by two facts. First, much of the work on vitamin B is now known to have been on a combined deficiency of vitamins B and G. Second, vitamin B exerts such a profound effect on food consumption that it is difficult to separate the effects of inanition from those of vitamin B starvation. In fact, the most recent work on this subject [100-102] indicates that practically all of the symptoms of vitamin B starvation are duplicated by withholding the food, at least in the case of pigeons. There is a very large literature on metabolism experiments with animals suffering from B avitaminosis, most of which is very difficult to interpret, because of the probable effects of starvation accompanied by vitamin deficiency. More clear-cut are the effects on the size of certain of the organs of the body or their functions. Most of the endocrine glands undergo atrophy, with the exception of the adrenals, which show hypertrophy. In the case of the reproductive organs, B avitaminosis appears to be specific in showing more rapid atrophy and cessation of oestrous in the female than would be accounted for by starvation. It must be admitted, however, that some of these results may have been compli-

[95] Williams, R. R., and Seidell, A., The Chemical Nature of the "Vitamines." II. Isomerism in Natural Antineuritic Substances, *J. Biol. Chem.*, 26 : 431–456 (1916).

[96] Williams, R. R., The Chemical Nature of the "Vitamines." III. The Structure of the Curative Modifications of the Hydroxypyridines, *J. Biol. Chem.*, 29 : 495–520 (1917).

[97] Williams, R. R., Vitamines from the Standpoint of Structural Chemistry, *J. Ind. Eng. Chem.*, 13 : 1107–1108 (1921).

[98] Harden, A., and Zilva, S. S., The Alleged Antineuritic Properties of α-Hydroxypyridine and Adenine, *Biochem. J.*, 11 : 172–179 (1917).

[99] Hofmeister, F., Zur Kenntnis der alkaloidischen Bestandteile der Reiskleie, *Biochem. Z.*, 103 : 218–224 (1920).

[100] Drummond, J. C., and Marrian, G. F., The Physiological Rôle of Vitamin B. Pt. I. The Relation of Vitamin B to Tissue Oxidations, *Biochem. J.*, 20 : 1229–1255 (1926).

[101] Kon, S. K., and Drummond, J. C., The Physiological Rôle of Vitamin B. Pt. III. Study of Vitamin B Deficiency in Pigeons, *Biochem. J.*, 21 : 632–652 (1927).

[102] Marrian, G. F., Baker, L. C., Drummond, J. C., and Woollard, H., The Physiological Rôle of Vitamin B. Pt. V. The Relation of Inanition to Vitamin B Deficiency in Pigeons, *Biochem. J.*, 21 : 1336–1348 (1927).

cated by vitamin G deficiency. The same may be said for the lowered resistance to certain bacterial infections which has been noted to accompany vitamin B deficiency.[103-104]

The effect of vitamin B deficiency on the appetite deserves further mention because of the peculiar fact that animals suffering from B avitaminosis appear to lose their appetite only for the vitamin B deficient food. This fact lends little support to the belief that vitamin B deficiency *per se* impairs the biochemistry and physiology of digestion. Beri-beri and polyneuritis, which may result from B avitaminosis, are still regarded as specific effects, inasmuch as they cannot be produced by starvation.

The profound effect of vitamin B on the appetite explains its classification as a growth-promoting vitamin. Attempts to assign the growth-promoting properties formerly associated with the complex to the G fraction are in error. Both vitamins are clearly required for growth, and each is therefore growth-promoting.

Very little is known regarding the origin of the antineuritic vitamin. The fact that yeast is one of the richest known sources of this factor, shows that it is synthesized by the very lowest forms of life. Bacteria also synthesize this vitamin.[105] The fact that the embryo of various seeds in many cases contains a higher concentration of vitamin B than the remainder of the seed, has suggested a relation of the vitamin to germination. No evidence has been found as yet that vitamin B is formed during germination.[106] The complete dependence of all animals,[107] so far tested, on their food for vitamin B indicates that its origin is confined exclusively to plants.

Vitamin B is fairly abundant in all animal products normally selected by man as a part of his dietary. Among the foods of plant origin, leaves and roots contain the vitamin, as do the whole seeds. In many cases, however, man has selected food preparations from the seeds which have had their vitamin B largely eliminated during the

[103] Werkman, C. H., Immunologic Significance of Vitamins. II. Influence of Lack of Vitamins on Resistance of Rat, Rabbit, and Pigeon to Bacterial Infection, *J. Inf. Dis.*, 32 : 255–262 (1923).

[104] Findlay, G. M., The Relation of Deprivation of Vitamin B to Body Temperature and Bacterial Infection, *J. Path. Bact.*, 26 : 485–495 (1923).

[105] Bechdel, S. I., Honeywell, H. E., Dutcher, R. A., and Knutsen, M. H., Synthesis of Vitamin B in the Rumen of the Cow, *J. Biol. Chem.*, 80 : 231–238 (1928).

[106] Harrow, B., and Krasnow, F., Feeding Experiments on Rats with Plants at Different Stages of Development. I. Experiments with Corn, *J. Metabolic Research*, 2 : 401–415 (1922).

[107] Sweetman, M. D., and Palmer, L. S., Insects as Test Animals in Vitamin Research. I. Vitamin Requirements of the Flour Beetle, *Tribolium confusum* Duval, *J. Biol. Chem.*, 77 : 33–52 (1928).

process of manufacture. This is true of all forms of degerminated cereals and of the flour made from them. Such products have other important dietary properties, but their deficiency in vitamin B should be clearly recognized.

Vitamin B may be extracted fairly easily from plant and animal materials. Yeast, rice polishings, or fat-free wheat embryo are the plant materials most commonly employed; they are relatively rich in this factor. Acidulated water, acidulated (*p*H 4.5) 20 per cent alcohol, 70 per cent methyl or ethyl or propyl alcohol or 70 per cent acetone have been used successfully, giving nearly complete extraction.

Vitamin B is adsorbed from its aqueous or slightly alcoholic aqueous solutions, the conditions most favorable for maximum adsorption depending upon the character of the adsorbent. For Lloyd's reagent the reaction is *p*H 4, for silica gel *p*H 5,[108] for Norit *p*H 7.[109] The release of vitamin B from its adsorbent is effected by changing the *p*H of the medium in which the adsorbent is suspended. For Lloyd's reagent most investigators use saturated barium hydroxide solution at a *p*H of ± 12, but Levene and van der Hoeven find that the maximum release occurs at *p*H 9–9.5, thus making it possible to employ other alkalies, so long as the *p*H is controlled. However, barium hydroxide has the advantage that the cation can be later removed with sulfuric acid. The same conditions apparently apply to the release of vitamin B from silica gel.[110] Norit, on the other hand, gives up its vitamin B in acid solution (*p*H2.5). This makes it possible to use Norit as a decolorizing agent with acid solutions of vitamin B (cf. Jansen and Donath).

Vitamin B forms insoluble compounds with phosphotungstic, tannic, picric, and picrolonic acids, each of which has been found valuable as a means of purification of the vitamin. Salts are also formed with the noble metals, silver, gold, and platinum, but not with mercury or lead in neutral or slightly alkaline solution. Mercuric sulfate and neutral or basic lead acetate are thus valuable non-vitamin precipitants in the purification of vitamin B concentrates. The use of silver sulfate (silver nitrate in sulfuric acid solutions) has been very common in the application of the Kossel and Kutscher[111] hexone base separation to the isolation of vitamin B. By using *p*H control during the neutralization of the vitamin silver solutions, Jansen and Donath were able to eliminate

[108] Levene, P. A., and van der Hoeven, B. J. C., The Concentration of Vitamin B, *J. Biol. Chem.*, 61 : 429–443 (1924).

[109] Kinnersley and Peters, *loc. cit.*

[110] Levene, P. A., and van der Hoeven, B. J. C., The Concentration of Vitamin B. II. *J. Biol. Chem.*, 65 : 483–489 (1925).

[111] Kossel, A., and Kutscher, F., Beiträge zur Kenntniss der Eiweisskörper, *Z. physiol. Chem.*, 31 : 165–214 (1900).

much vitamin-free material at *p*H 4.5 and recover most of the vitamin at *p*H 6.5.

The greater part of the original vitamin B in natural foods is lost during the isolation, even with the best chemical control so far devised. This is probably due in large part to the instability of this factor, especially in the presence of alkali. This has been known since the early observations of Chamberlain, Vedder, and Williams, and has been one of the chief obstacles in the isolation of this factor. Acids, on the other hand, exert relatively little destructive action towards vitamin B. These facts lend support to Williams' hypothesis of a structure capable of keto-enol isomerism, the keto being the stable active form.

The stability of vitamin B in solution or in its natural state in foods is influenced by the temperature as well as by the reaction of the medium. Natural foods, particularly grains and cereals, are known to lose their antineuritic properties when heated at 120° C. for one-half hour or more. The destruction is undoubtedly appreciable at 100° C., when the reaction is neutral or slightly alkaline. The experiment of Sherman and Burton,[112] using weight maintenance of rats as an index, can probably be interpreted as showing the rate at which vitamin B is destroyed at 100° C. at different reactions. Heating for one hour at *p*H 4.28 and *p*H 5.20 destroyed only about 10 per cent of the vitamin, but at *p*H 7.9 the loss was 30 per cent and at *p*H 9.2, 60–70 per cent. It would seem that the ordinary home cooking of fruits and vegetables does not bring about a serious diminution in the antineuritic properties of food, but pressure cooking is likely to be somewhat more destructive.

Vitamin G.—A large amount of fairly conclusive circumstantial evidence gradually accumulated and was interpreted as indicating that the growth-promoting and antineuritic properties of food, food fractions and extracts from the same, resided in a single vitamin. For example, (1) the distribution of the two properties in nature was very similar, (2) various extracts and preparations from natural foods having antineuritic properties were likewise growth-promoting, (3) growth failure was almost invariably followed by polyneuritis, (4) the solubility and adsorption properties, and behavior toward precipitants appeared to be identical for the substance having antineuritic and growth-promoting effects, (5) heat, or heat plus alkalies destroyed both the antineuritic and growth-promoting properties of foods.

Most of the attempts to discover the possible existence of more than one component in the factor formerly called vitamin B fell short of con-

[112] Sherman, H. C., and Burton, G. W., Effect of Hydrogen Ion Concentration upon the Rate of Destruction of Vitamin B upon Heating, *J. Biol. Chem.*, 70 : 639–645 (1926).

clusive proof, because comparisons were made between the requirements of rats and pigeons, as well as between growth-promoting and antineuritic properties. Much more conclusive[113-114] were the findings regarding the comparative growth-promoting and antineuritic properties of yeast concentrate or yeast and grain when studied with the same species of animal, indicating that at least two "B" components in foods were required for growth. The conclusive proof of the existence of vitamin G rests on the observations[115-116] that normal growth of rats requires not only the antineuritic vitamin B but in addition some substance still residing in autoclaved yeast which is no longer antineuritic. Particularly significant is the finding by Goldberger and associates that the heat-stable factor in yeast has the specific property of curing and preventing human pellagra, and also a syndrome in dogs, called black tongue, that seems to be analogous to pellagra. This makes it possible to ascribe to vitamin G a specific property that distinguishes it from all other vitamins. The growth-promoting and antipellagric properties of vitamin G thus became analogous to the growth-promoting and antineuritic properties of vitamin B.

The discovery of vitamin G is too recent to warrant any extended statements regarding its functions in nutrition. So far as is known at present, the growth-promoting properties are due indirectly to the effect on appetite, as in the case of vitamin B. For rats, the lesions ascribed to a deficiency of vitamin G are largely epidermal.[117-118] There is a dermititis of the ears, neck, chest, legs, and paws, with desquamation on healing. There is more or less depilation, usually beginning on the head and at times extending over most of the body. A type of ophthalmia, with inflamed eyelids and sunken eyes, is also described. In black tongue of dogs there is some localized dermititis, but the characteristic

[113] Kinnersley, H. W., and Peters, R. A., Antineuritic Yeast Concentrates. I. *Biochem. J.*, 19 : 820-826 (1925).

[114] Hauge, S. M., and Carrick, C. W., A Differentiation between the Water-Soluble Growth-Promoting and Antineuritic Substances, *J. Biol. Chem.*, 69 : 403–413 (1926).

[115] Smith, M. I., and Hendrick, E. G., Some Nutrition Experiments with Brewer's Yeast, with Especial Reference to Its Value in Supplementing Certain Deficiencies in Experimental Rations, *U. S. Public Health Reports*, 41 : 201–207 (1926).

[116] Goldberger, J., Wheeler, G. A., Lillie, R. D., and Rogers, L. M., A Further Study of Butter, Fresh Beef and Yeast as Pellagra Preventives with Consideration of the Relation of Factor P-P of Pellagra (and Black Tongue of Dogs) to Vitamin B, *U. S. Public Health Reports*, 41 : 297–318 (1926).

[117] Goldberger, J., and Lillie, R. D., A Note on an Experimental Pellagralike Condition in the Albino Rat, *U. S. Public Health Reports*, 41 : 1025–1029 (1926).

[118] Chick, H., and Roscoe, M. H., The Dual Nature of Water-Soluble Vitamin B. II. The Effect upon Young Rats of Vitamin B_2 Deficiency and a Method for the Biological Assay of Vitamin B_2, *Biochem. J.*, 22 : 790–799 (1928).

effect is the inflammation and congestion of the tongue and the entire mucosa of the mouth and lips. In man the characteristic symptoms of vitamin G deficiency are those of pellagra. Both the dermititis seen in the rat and the soreness of the mouth seen in dogs occur, together with more or less nervous symptoms involving the spinal cord and central nervous system.

Although vitamin G seems to be distributed in nature in conjunction with vitamin B, it is evident that the relative distribution of these two factors varies considerably. In general, it seems that seeds, and grains, and roots are relatively poor in vitamin G, but relatively rich in vitamin B. The reverse is true for foods of animal origin, such as milk, meat and eggs. Yeast, however, usually appears to be rich in both vitamins, but like other plant materials is relatively richer in vitamin B. These comparisons are based on the assumption that the growing animal requires equal quantities of vitamins B and G, the validity of which cannot be proven until the factors are isolated and the absolute requirements determined.

Nothing is known, as yet, regarding the probable chemical nature of vitamin G. It seems obvious that it will be found to resemble vitamin B, inasmuch as the two vitamins possess many properties in common. Vitamin G appears to form compounds with the alkaloid reagents and with silver, since many of the crude vitamin " B " concentrates prepared have evidently been mixtures of the antineuritic and antipellagric vitamins. Vitamin G is adsorbed by Lloyd's reagent and silica gel. By interposing a collodion membrane between yeast extracts and Lloyd's reagent, Williams and Waterman[119] appear to have prevented the adsorption of vitamin G and to have effected the adsorption of vitamin B only. Levene[120] finds that silica gel adsorbs vitamin B preferentially from yeast extracts at *p*H 3, thus furnishing a preliminary basis for the separation of the factors.

The outstanding difference between vitamins G and B is the great heat stability of the former, as well as its stability towards alkalies. This evidently represents a difference between the chemical structure of the two vitamins, the nature of which will be revealed by future investigation. At present the chief advantage of this difference lies in the possibility of testing foods for their vitamin G content without the food itself being antineuritic. The latter vitamin may be supplied in the form of

[119] Williams, R. R., and Waterman, R. E., The Tripartite Nature of Vitamin B, *J. Biol. Chem.*, 78 : 311–322 (1928).

[120] Levene, P. A., The Concentration of Vitamin B. IV. On the Concentration and the Separation of the Two Components of Vitamin B, *J. Biol. Chem.*, 79 : 465–470 (1928).

concentrate, such as that of Kinnersley and Peters. By using autoclaved yeast as a supplement to natural foods at various levels in otherwise complete diets, one can also determine whether vitamin B or G is the limiting vitamin (cf. Sherman and Axtmayer[121]).

VITAMIN C.—The experimental proof of the existence of an antiscorbutic vitamin is furnished by a modern interpretation of the extensive investigations of Holst,[122] Holst and Frölich,[123-124] and Fürst.[125] First, in the experimental production of scurvy in guinea pigs, and second, through a classic study of experimental scurvy, these experimenters demonstrated for the first time that the long-known relation between diet and scurvy could not be explained by any of the known dietary factors. The rapid development of the vitamin hypothesis which almost immediately followed these experiments thus made it easy to classify the antiscorbutic factor as a vitamin. Certain difficulties of technic in producing experimental scurvy and a clear understanding of the proper relation of vitamins A and B to nutrition, as well as an appreciation of the differences in susceptibility of different species of animals to the disease, prevented the general acceptance of a vitamin etiology of scurvy until about 1919.[126]

Susceptibility to scurvy is apparently confined to man, monkeys, and guinea pigs. In these species the disease appears to be primarily one of capillary hemorrhage. Abnormalities of the bones and teeth occur, resulting in great fragility of these structures. Hypertrophy of the adrenals is another characteristic effect. For a more complete description of the pathological changes resulting from vitamin C deficiency and for a bibliography of the subject, consult Hess,[127] and McCollum.[128]

[121] Sherman, H. C., and Axtmayer, J. H., A Quantitative Study of the Problem of the Multiple Nature of Vitamin B, *J. Biol. Chem.*, 75 : 207–212 (1927).

[122] Holst, A., Experimental Studies Relating to "Ship-Beri-Beri" and Scurvy, *J. Hyg.*, 7 : 619–633 (1907).

[123] Holst, A., and Frölich, T., On the Etiology of Scurvy, *J. Hyg.*, 7 : 634–671 (1907).

[124] Holst, A., and Frölich, T., Über experimentellen Skorbut, *Z. Hyg. Infektionskrankh.*, 72 : 1–120 (1912).

[125] Fürst, V., Weitere Beiträge zur Ätiologie des experimentellen Skorbuts des Meerschweinchens, *Z. Hyg. Infektionskrankh.*, 72 : 121–154 (1912).

[126] Drummond, J. C., Note on the Rôle of the Antiscorbutic Factor in Nutrition, *Biochem. J.*, 13 : 77–80 (1919).

[127] Hess, A. F., Scurvy, Past and Present, J. B. Lippincott Company, Philadelphia (1900).

[128] McCollum, E. V., Our Present Knowledge of the Vitamins, Lectures on Nutrition, Mayo Foundation, pp. 176–179. W. B. Saunders Company, Philadelphia (1925).

Bezssonoff[129-130] has presented experiments showing that vitamin C acts as a regulator of the elimination of the toxic substances that are normal waste products of digestion, chiefly those of the phenol type. This is believed to be greatly augmented by the inanition that usually becomes increasingly severe during the progress of the disease.

No complete list of antiscorbutic foods is available. However, vitamin C is primarily a plant product. Most animal tissues or secretions containing the vitamin must derive it in part from the food. The presence of vitamin C in the liver of rats, calves, and chickens, and in the milk of cows, all fed vitamin C deficient diets, may be interpreted as indicating the synthesis of this vitamin by the animals from its precursor in seeds and grains. The site of this synthesis is as yet unknown.

Among plants, vitamin C is present more or less abundantly in the fresh young leaves, stems, roots, bulbs, fruits, and immature seeds. Freshly pressed juices from all of these plant parts are rich in vitamin C. According to McCollum,[131] tomatoes picked green and ripened by keeping are poor in vitamin C in contrast with the high content of the factor in the naturally ripened fruit.

The synthesis of vitamin C in plants appears to be associated with the fundamental processes of germination and growth. Fürst discovered that germinated seeds are strongly antiscorbutic in contrast to the dry dormant seeds. The vitamin appears in the seed as soon as active growth begins, and is detectable before any appreciable growth of the embryo can be observed. Oxygen is necessary, but not light or photochemical action. The facts supporting these statements are: (1) Vitamin C synthesis is lost when the power of germination is destroyed by grinding. (2) The mere soaking of most seeds in water gives them some antiscorbutic value.[132] (3) Germination in the dark is as effective as germination in the light (cf. Chick and Delf[133]). (4) Aerobic conditions must prevail in order for seeds to generate vitamin C during soaking in water (cf. Honeywell and Steenbock[134]). Although light is not

[129] Bezssonoff, N., Une méthode nouvelle pour caractériser l'action physiologique immédiate des vitamines hydrosolubles, *Bull. soc. chim. biol.*, 10 : 1179–1198 (1928).

[130] Bezssonoff, N., Les effets physiologiques immédiats de l'avitaminose C, *Bull. soc. chim. biol.*, 10 : 1199–1212 (1928).

[131] McCollum, E. V., Water-Soluble Vitamins, Columbia University Press, New York (1927).

[132] Fürst, V., Weitere Beiträge zur Ätiologie des experimentellen Skorbuts des Meerschweinchens, *Z. Hyg. Infektionskrankh.*, 72 : 121–154 (1912).

[133] Chick, H., and Delf, E. M., The Anti-Scorbutic Value of Dry and Germinated Seeds, *Biochem. J.*, 13 : 199–218 (1919).

[134] Honeywell, E. M., and Steenbock, H., The Synthesis of Vitamin C by Germination, *Am. J. Physiol.*, 70 : 322–332 (1924).

necessary for vitamin C synthesis during germination, the vitamin C concentration in seedlings is increased greatly by exposure to light (cf. Heller[135]).

Water is the universal solvent for vitamin C. Ethyl and methyl alcohol also dissolve the vitamin. Butyl alcohol does not. The vitamin is not soluble in any of the ordinary fat solvents except acetone. It cannot be extracted from plants, however, by this solvent.

Vitamin C is not adsorbed by fuller's earth or by colloidal ferric hydroxide under the conditions which adsorb vitamins B and G. These facts make it possible to separate vitamin C from the other water-soluble vitamins, both for feeding experiments and for isolation of the C factor (cf. Harden and Zilva[136]).

Vitamin C diffuses readily through parchment and collodion membranes. Connell and Zilva[137] found that the differential dialysis of vitamin C, invert sugar, and nitrogenous substances from decitrated lemon juice through collodion membranes of varying porosity revealed a diffusion rate for vitamin C resembling that of the sugar molecule.

Vitamin C is not precipitated from its solutions by alcohol or by acid lead acetate, but is precipitated by basic lead acetate. The vitamin may be freed from its lead salt without destroying the vitamin. These properties have proved exceptionally useful in the concentration of vitamin C during attempts to isolate the vitamin and determine its chemical nature.

Probably more has been written about the instability of vitamin C to heat than about any of its other properties. A large amount of early evidence pointed to the conclusion that vitamin C is readily destroyed by heat, but as the data accumulated it became evident that other factors, for example, hydrogen ion concentration, the presence of other materials in the solution, and oxygen, greatly modified the destructive effects of heat. LaMer, Campbell, and Sherman[138] regard the heat destruction as an intramolecular oxidation and reduction. The temperature coefficient of the reaction is of a low order, being about 1.2 for each ten-degree

[135] Heller, V. G., Vitamin Synthesis in Plants as Affected by Light Source, *J. Biol. Chem.*, 76 : 499–511 (1928).

[136] Harden, A., and Zilva, S. S., The Differential Behaviour of the Antineuritic and Antiscorbutic Factors towards Adsorbents, *Biochem. J.*, 12 : 93–105 (1918).

[137] Connell, S. J. B., and Zilva, S. S., The Differential Dialysis of the Antiscorbutic Factor. II. *Biochem. J.*, 18 : 641–646 (1924).

[138] LaMer, V. K., Campbell, H. L., and Sherman, H. C., The Effect of Temperature and the Concentration of Hydrogen Ions upon the Rate of Destruction of Antiscorbutic Vitamin (Vitamin C), *J. Am. Chem. Soc.*, 44 : 172–181 (1922).

rise in temperature (cf. Delf[139]). The importance of oxidation in the heat destruction of vitamin C is shown by the fact that an alkaline decitrated lemon juice (*p*H 12) may lose 80 per cent of its activity on standing for one hour exposed to the air at room temperature, but lose none of its activity in twenty-four hours under anaerobic conditions (cf. Zilva[140]). The practical application of these facts is found in the work of Kohman, Eddy, and Carlsson,[141-142] who show that vitamin C in fruit and vegetables may be preserved during heat processing of the canned foods, provided cell oxygen is used up prior to the sealing in the cans, or open-kettle cooking is eliminated.

Vitamin C is much more stable in acid solution than in a neutral or alkaline medium. However, even acid fruit juices require protection from oxidation (cf. Lepkovsky, Hart, Hastings, and Frazier[143]).

Vitamin C is unaffected by ultraviolet rays, provided it is at the same time protected from oxidation (cf. Zilva[144]).

Destruction of vitamin C occurs during the bacterial acid fermentation of foods originally rich in this factor. Sauerkraut and corn silage are not antiscorbutic.[145] Vitamin C is not lost, however, during yeast fermentation[146] or in the presence of pentose-fermenting organisms or proteolytic bacteria.[143]

The antiscorbutic value of cooked and canned foods has been shown to be determined by (1) the original vitamin content, (2) the hydrogen ion concentration of the material, (3) the oxidation potential of the food, both internal and external, and (4) the temperature and time of cooking

[139] Delf, E. M., Effect of Heat on the Antiscorbutic Accessory Factor of Vegetable and Fruit Juices, *Biochem. J.*, 14 : 211–228 (1920).

[140] Zilva, S. S., The Influence of Reaction on the Oxidation of the Antiscorbutic Factor in Lemon Juice, *Biochem. J.*, 17 : 410–415 (1923); The Antiscorbutic Fraction of Lemon Juice. VII. *Biochem. J.*, 22 : 779–785 (1928).

[141] Kohman, E. F., Eddy, W. H., and Carlsson, V., Vitamins in Canned Foods. II. The Vitamin C Destructive Factor in Apples, *Ind. Eng. Chem.*, 16 : 1261–1263 (1924).

[142] Eddy, W. H., Kohman, E. F., and Carlsson, V., Vitamins in Canned Foods. III. Canned Spinach, *Ind. Eng. Chem.*, 17 : 69–74 (1925).

[143] Lepkovsky, S., Hart, E. B., Hastings, E. G., and Frazier, W. C., The Effect of Fermentation with Specific Microorganisms on the Vitamin C Content of Orange and Tomato Juice, *J. Biol. Chem.*, 66 : 49–56 (1925).

[144] Zilva, S. S., The Action of Ultra-Violet Rays on the Accessory Food Factors, *Biochem. J.*, 13 : 164–171 (1919).

[145] Ellis, N. R., Steenbock, H., and Hart, E. B., Some Observations on the Stability of the Antiscorbutic Vitamine and Its Behavior to Various Treatments, *J. Biol. Chem.*, 46 : 367–380 (1921).

[146] Zilva, S. S., The Antiscorbutic Fraction of Lemon Juice. I and II. *Biochem. J.*, 18 : 182–185; 632–637 (1924).

or processing. It appears that home cooking is likely to be always destructive of the vitamin C content of foods, but that the commercial canner is in a position to produce canned fruits and vegetables of a high antiscorbutic value.

Essentially the same problems are involved in the preservation of vitamin C during drying of foods, as in cooking and canning. With modern drying machinery, involving vacuum processes, it appears that it should be possible to commercially dry fruit juices and vegetables, and at the same time retain much of the original antiscorbutic value. Dried fruits, however, require further protection in sealed vacuum containers. For a more extended account of the successes and failures that have accompanied drying of antiscorbutic vegetables and fruit juices, including commercial drying, laboratory drying, and sun drying, consult McCollum and Simmonds.[147]

Vitamin C has not yet been isolated, so that its chemical nature is as yet undetermined. The concentrated preparations secured by various workers indicate that it is free from nitrogen and consists of the elements, carbon, hydrogen, and oxygen alone. Bezssonoff[148] reports having secured hygroscopic crystalline needles, melting at 47° C, which protected guinea pigs against scurvy in daily dosages of less than two milligrams. If this material was actually vitamin C, it is evident that the absolute requirement for this factor by animals whose normal existence depends upon its presence in their food is very much greater than for any of the other vitamins.

Two methods have been developed for concentrating vitamin C. Bezssonoff[149] presses the juice from cabbage under hydraulic pressure, clarifies the juice with acid lead acetate, and then precipitates the vitamin from the filtrate by bringing the solution to a *p*H of 8.2 with sodium hydroxide. The precipitate is dissolved in acetic acid, reprecipitated with alkali three times, and then deleaded. Further purification is effected by concentration *in vacuo* and resolution in distilled water repeated four times, by the use of alcohol to remove impurities and by extracting the final dry residues with absolute alcohol and acetone in successive operations.

Zilva[150] decitrates lemon juice with calcium carbonate, ferments the

[147] McCollum, E. V., and Simmonds, N., The Newer Knowledge of Nutrition, Third Edition, pp. 190–193, Macmillan Company, New York (1925).

[148] Bezssonoff, N., Quelques données sur la nature du principe antiscorbutique dit vitamine C, *Compt. rend.*, 180 : 970–972 (1925).

[149] Bezssonoff, N., Sur une préparation antiscorbutique et sur le rôle de la vitamine A dans le scorbut expérimental, *Bull. soc. hyg. aliment.*, 11 : 14–38 (1923).

[150] Zilva, S. S., The Antiscorbutic Fraction of Lemon Juice. II. *Biochem. J.*, 18 : 632–637 (1924).

sugars with yeast in an atmosphere of carbon dioxide, removes impurities by adding absolute alcohol, concentrates the vitamin-containing filtrate *in vacuo*, precipitates the vitamin with basic lead acetate, and deleads the precipitate, thus freeing the vitamin. It is stated that the solution of vitamin C thus obtained, when made up to the original volume of juice contains only 0.03 per cent to 0.07 per cent solids, and is as antiscorbutic as the original juice.

CHAPTER XXXV

ENZYMES

Catalysis.—It has long been known that the velocity of certain reactions can be increased by the presence of relatively small amounts of certain substances.

For example, hydrogen and oxygen gases do not combine at a measurable speed at ordinary temperature, but when a mixture of such gases is passed over platinum black, combination takes place, the reaction proceeding at such a velocity that the platinum black may actually become incandescent. A similar reaction has been used for a number of years in the industry for the synthesis of sulfur trioxide in the manufacture of sulfuric acid and is known as the contact process. Finely divided platinum has the property of greatly speeding up the decomposition of hydrogen peroxide.

Substances which have this remarkable power of hastening a chemical reaction are called catalytic agents, and their action is known as catalysis, from the Greek κατα meaning "down," and λυο meaning "to loosen," literally a down-loosening which has come to mean the hastening of a chemical reaction.

Until rather recently it has been generally agreed that there are two properties common to all catalysts, (1) that the catalyst does not initiate a new reaction but merely changes the rate of a reaction already in progress, and (2) that a catalyst does not appear in the final products of the reaction which is accelerated or that it is not used up in the process of the reaction. Within the last few years there has been a revision of opinion in regard to the first hypothesis, *i.e.*, that a catalyst is incapable of initiating a new reaction. According to the old belief the explanation for the speeding up of the reaction between the gases, hydrogen and oxygen, was that the reaction proceeded at room temperature, but the rate of the reaction was essentially negligible and could not be detected within measurable time. The newer conception is that *a catalyst may actually initiate a chemical reaction.* This viewpoint is excellently summed up by Reid[1] in the statement, "The old conception according to which the catalyst contributes only its presence and takes no part in

[1] Reid, E. E., Fifth Report of the Committee on Contact Catalysis, *J. Phys. Chem.*, 31 : 1121–1149 (1927).

the reaction has been given up. Zelinsky[2] remarks: ' My observations on catalysis extending over several years have brought me to the same view of catalytic phenomena as was expressed by Mendelejeff[3] long ago in such a simple and original form, a view which later Raschig[4] and recently it seems Bodenstein[5] have adopted. In the contact processes with carbon compounds, the catalyst does not determine the reaction simply by its presence but by taking the rôle of an active principle in the process; its surface energy produces far-reaching alterations in the substances which come into contact with it.' "

Boswell and Dilworth,[6] in discussing the mechanism of catalysis by aluminium oxide, go a step further, and after discussing the nature of the surface film surrounding the aluminium oxide particle, state (p. 1492), " This all points to the conclusion that this catalyst functions by means of a surface film of water and that this film is the real seat of the catalysis. The marked stability of the film indicates that it is present in a special condition such as positively charged hydrogens and negatively charged hydroxyls, alternating with each other and completely enveloping each aluminium oxide particle." Their conception is an *orientated adsorption* such as is shown in Fig. 65. After further discussing certain specific reactions catalyzed by aluminium oxide, Boswell and Dilworth state (p. 1495), " The catalyst from this point of view does not accelerate a reaction already in progress . . . but actually initiates the change . . . "

These modifications of the older conception are of extreme importance and afford a logical explanation for the behavior of a catalyst. According to the older viewpoint the catalyst was looked upon as a mysterious chemical compound which in some way speeded up a reaction. According to the newer viewpoint a catalyst is looked upon as a source of *surface energy,* the chemical nature of the catalyst being relatively unimportant providing that the space configuration of the atoms in the surface of the catalyst are such as to cause certain oriented adsorption relationships and the surface of the catalyst is in such a state as to contribute a given quantity of surface energy to the system. It has been generally recognized that many substances of the most diverse chemical nature may be

[2] Zelinsky, N. D., Über die Inaktivierung der Katalysatoren bei den Umwandlungen von Kohlenstoffverbindungen, *Ber.*, 59 : 156–162 (1926).

[3] Mendelejeff, D., *J. Russ. Chem. Soc.*, 18 : 8–18 (1886) (in Russian) cf. also Notiz über Contactwirkungen, *Ber.*, 19 : 456–463 (1886).

[4] Raschig, F., Gedanken über Katalyse, *Z. angew. Chem.*, 19 : 1748–1763 (1906). Cf. also Bredig, G., Bemerkungen zu Raschig's " Gedanken über Katalyse," *Z. angew Chem.*, 19 : 1984–1987 (1906).

[5] Bodenstein, M., Ein Beitrag zur Theorie der katalytischen Hydrierung durch Platin, *Ann.*, 440 : 177–185 (1924).

[6] Boswell, M. C., and Dilworth, H. M., On the Mechanism of Catalysis by Aluminium Oxide, *J. Phys. Chem.*, 29 : 1489–1506 (1925).

used to catalyze a given reaction. The difficulty in the past has been to explain such catalysis on the basis of the diverse chemical nature of the catalysts which were involved. Under the new viewpoint where only surface energy forces and oriented adsorption are regarded as important, it is very easy to see how many diverse substances in the proper physical state may contribute identical amounts of surface energy and actually accelerate reactions at the same rate. Similarly this viewpoint affords a logical explanation of the newer belief that a catalyst may initiate a reaction, which is not already in progress, by contributing to a system the determining amount of surface energy which is necessary to initiate a given reaction.

A catalyst, therefore, must be looked upon as a substance which alters the rate of reaction, but it may be either in the direction of acceleration or of retardation. The reaction may be one that in the absence of a catalyst proceeds relatively rapidly or proceeds so slowly that it requires special proof to show that it is taking place at all, or at least in certain instances is not, in the absence of the catalyst, detectable by any means as yet at the disposal of the chemist.

Catalysts do not appear, as a rule, in the end products of the reaction, but there is no doubt that in many cases, if not in all, the catalytic surface has actually entered into the reaction at some stage or other, or at least that an oriented adsorption has taken place upon the surface of the catalyst and in this way the reacting molecules have been brought within the sphere of chemical attraction and chemical reactivity. If the products of the reaction are less strongly adsorbed upon the catalyst than are the reacting materials, the reaction products will leave the surface of the catalyst, allowing additional adsorption of the reacting materials, which in turn will combine, thus speeding up the rate of synthesis of the final products.

Although the degree of acceleration of a reaction is, within certain limits, proportional to the concentration of the catalyst present, the final equilibrium appears to be independent of the amount of catalyst. It is surprising how minute a quantity of a catalyst is capable of perceptible catalytic activity. For example, colloidal platinum will decompose a million times its weight of hydrogen peroxide. Since the addition of a catalyst increases the rate of a chemical reaction, it follows that the time required to produce a definite equilibrium must vary with the amount of catalyst which is present.

Water is one of the most important catalytic agents which are known. In certain instances the presence of water accelerates a reaction, in other instances retards or inhibits a reaction, especially when water is a product of the reaction. Perfectly dry ammonia and hydrogen chloride gases

will not combine with a measurable speed, but in the presence of traces of water the reaction is almost instantaneous. Chlorine and hydrogen gases when pure will not combine at measurable speed to form hydrogen chloride, but in the presence of traces of water the reaction is greatly accelerated. As we shall see later, water enters into many of the reactions characteristic of the biocatalysts.

Enzymes as Catalysts.—The catalytic agents of cells and of the constituents of living organisms are known as enzymes, a word derived from the Greek, meaning literally "in yeast" or "in leaven." An enzyme thus may be regarded as an organic catalytic agent found in living matter. These catalytic agents are very numerous, and it is to them that we ascribe the chemical reactions which occur in living protoplasm. The food of plants and of animals, the proteins, carbohydrates, fats, etc., is in general valueless unless it is brought into a condition suitable for assimilation and translocation. The starch of the leaf must be rendered soluble before it can be transported to other parts of the plant. The starch of the potato tuber must similarly be rendered soluble before it can be used for the nutrition of the young shoots. The starch of our foods must be rendered soluble before it will pass from the intestinal tract into the blood stream and from the blood stream to the tissues of the body. The glycogen which is stored in the liver as a reserve source of energy must similarly be rendered soluble by the action of enzymes before it can be utilized as a source of energy by the organism. Similarly the proteins, the fats, the lipides, and, in general, all of the energy- and tissue-building constituents of both plants and animals must be acted upon by one or more enzymes before they are rendered available for the use of the organism. Thus, it is seen that enzyme action is a strategic center of vital activity.

In 1811, Kirchhoff[7] noted that starch was transformed by mineral acids into glucose, and he demonstrated that no acid was consumed in the process. This then is an example of a chemical reaction catalyzed by hydrogen ions. In 1833, Payen and Persoz[8] made the discovery which has had far-reaching consequences, *i.e.*, that germinating seeds contain a peculiar contact substance which transforms starch into sugar. This substance they called *diastase*, a name which still persists in the literature, although *amylase* appears preferable. Inasmuch as diastase is concerned with the hydrolysis of starch and is the active principle in malt, diastase

[7] Kirchhoff, G. S., Ueber die neue von Kirchhoff entdeckte Zuckergewinnung, *Schweigger's J. f. Chem. u. Phys.*, 4 : 108–110 (1811).

[8] Payen and Persoz, Mémoire sur la diastase. Les principaux produits de ses reactions, et leurs applications aux arts industriels, *Ann. chim. phys.*, 53 : 73–92 (1833).

has probably been studied more extensively than has any other enzyme. Therefore, the real beginning of enzyme study dates back to the observations of Payen and Persoz.

It is known that a great number of chemical reactions are what are termed reversible reactions. An example of such a reaction is the formation of acetic acid and alcohol from ethyl acetate and water:

$$\text{Ethyl acetate} + \text{water} \rightleftharpoons \text{ethyl alcohol} + \text{acetic acid.}$$

The characteristic of a reaction of this kind is that it progresses in one direction or the other until a certain equilibrium specific to the reaction and to the concentration of the reacting materials is established. Any alteration in concentration of the reacting substances is immediately followed by a change to a new equilibrium. The velocity of the reaction moreover may be accelerated by the presence of either hydrogen or hydroxyl ions which, although they do not affect the nature of the final products, accelerate the process and alter the position of equilibrium. In this instance, either hydrogen or hydroxyl ions may be regarded as catalysts.

In the plant or in the animal organism many reactions are known to be of the above type, as for instance the hydrolysis of starch into sugar and the synthesis of starch again from the sugar. Outside of the living organism the hydrolysis of starch by purely chemical processes takes place only at an elevated temperature or in the presence of an appreciable concentration of hydrogen ions. Within the living organism or in the presence of enzymes the hydrolysis occurs at ordinary temperatures with considerable rapidity and is controlled by the amyloclastic enzymes. Amylase, therefore, is a catalyst produced by the living organism. The extraction of amylase and the demonstration of its catalytic action *in vitro* is, of course, a perfectly simple experiment, but the synthesis of starch from sugar by the same enzyme has not as yet been carried out *in vitro*. It is reasonable to assume, however, that amylase controls the process in both directions, and there are other instances where the synthetic power of enzymes has been demonstrated *in vitro* under special conditions, for example, the synthesis of glucosides by emulsin which has already been noted. It is, however, not necessary to postulate that all enzyme reactions are reversible reactions. It is entirely probable that in some instances one enzyme may control the synthetic process, and another, and a different enzyme, the process whereby the substrate is broken down.

Fermentation.—The formation of alcohol and carbon dioxide from sugar, on account of the effervescence or apparent boiling due to the escape of carbon dioxide, was early called fermentation from the Latin,

ferveo, to boil. When Pasteur showed that this process was due to the presence of a living organism, diastase and other biocatalysts analogous to diastase were called " soluble or unorganized ferments " to distinguish them from living organisms, such as yeasts and certain bacteria which were termed " organized ferments." On account of the confusion of the terms it was suggested by Kühne, in 1878, that the " soluble ferment " should be termed " enzyme " which at that time signified that something in yeast which caused the fermentation of sugar.

It was definitely shown by Buchner[9] that yeast did contain an enzyme which fermented sugar in the absence of the living cells. The yeast was ground so that the contents of the cells could be extracted by pressure. The liquid so obtained, freed by filtration from every trace of living cells or their fragments, still possessed the property of causing alcoholic fermentation analogous to, although at a lower rate than that caused by the living yeast. He also demonstrated that the alcohol-forming substance was soluble in water, precipitable by alcohol, and very easily destroyed by heat.

Definition of Enzymes.—Waldschmidt-Leitz[10] defines enzymes as " *definite material catalyzers of organic nature with specific powers of reaction, formed indeed by living cells, but independent of the presence of the latter in their operation.*" This definition appears to be fairly satisfactory, although it should probably have appended to it the phrase, *and, when in the moist state, readily destroyed by heat.*

Occurrence of Enzymes.—Enzymes may occur in the secretions of living organisms, and as such act outside of the normal protoplasmic mass. Such enzymes are known as extracellular or secretion enzymes, and in the living organism occur in secretions which appear to pass through the living cell membrane. Such enzymes normally act outside of the cells which produce them. Ptyalin, the amylase of the saliva, pepsin, the protease of the gastric juice, and sucrase, the inverting enzyme of yeast, are typical examples of extracellular enzymes. Preparations of such enzymes are rather readily obtained in quantity, and most of our knowledge of enzymatic behavior has been gained through a study of the behavior of extracellular enzymes.

The intracellular enzymes appear to be non-diffusible through the cell membrane. They are not excreted by the living organism but instead act inside of the cell. In some instances it is possible to destroy the protoplasm without inactivating the enzymes. In other instances

[9] Buchner, E., Alkoholische Gärung ohne Hefezellen, Vorlauf, Mitt., *Ber.*, 30 : 117–124 (1897); Zweite Mitt., *Ber.*, 30 : 1110–1113 (1897).

[10] Waldschmidt-Leitz, E., Enzyme Actions and Properties, translated and extended by R. P. Walton, John Wiley and Sons, Inc., New York (1929).

it has not as yet been possible to prepare enzyme preparations which reproduce *in vitro* the chemical reactions characteristic of living organisms and which reactions we have every reason to believe are due to the presence of intracellular enzymes.

There are three general methods available for the preparation of intracellular enzymes. Buchner proposed the method of grinding the cellular material with sand and fuller's earth and placing this ground mass in a hydraulic press, certain of the intracellular enzymes being released by the grinding process and appearing in the press liquor. In this way he demonstrated the presence of zymase as the intracellular fermenting enzyme of yeast. It should be noted, however, that this method permits only the demonstration of intracellular enzymes which are soluble in water or which disperse in water to form colloidal sols. Any enzymes which may be insoluble in water or which are strongly adsorbed upon the cell wall fragments or upon the sand or fuller's earth surfaces would not appear in the extract.

Rowland modified the method of Buchner in that he froze the cellular mass and then ground the frozen material in a mill. Wiechowski later modified this method by pulping the tissue, pressing it through a sieve, drying it at a low temperature, and then grinding the dried material with toluene in a paint mill. The fine powder was removed from the toluene suspension and dried. All cells had been disintegrated by this method, and the dry cell-free powder was used as an enzyme preparation.

The Classification of Enzymes.—We have already noted many instances of enzyme action in connection with the proteins, carbohydrates, glucosides, fats, lipides, and pigments, all of which serve as substrates and are altered by the action of one or more enzymes. Accordingly in this chapter we will not include a discussion of the specific enzymes in relation to specific reactions. The enzyme literature is exceedingly voluminous, as is evidenced by the recent compilations of Oppenheimer[11] and Euler.[12]

The great majority of enzymes are those which may be classed as hydrolyzing enzymes, indicating that the elements of water enter into the chemical reaction and that the reactions usually take place in the presence of an excess of water. This factor is sometimes overlooked as an important factor in enzyme phenomena, whereas in reality the relative abundance of water is probably an extremely important factor in governing the direction and position of the final equilibrium.

[11] Oppenheimer, C., Die Fermente und ihre Wirkungen, Fifth Edition, 3 Vols., Georg Thieme, Leipzig (1925–1929).

[12] Euler, Hans von, Chemie der Enzyme, Third Edition, 2 Vols., J. F. Bergmann, Munich (1925–1927).

In the following classification have been listed all, or nearly all, of the enzymes for which adequate characterizations have been given, together with the substrate upon which the enzyme acts and the end products of the reaction in so far as the end products are actually known. It is entirely probable that certain of the enzymes which, with our present knowledge, have been given specific names may in reality be composed of a mixture of enzymes. It is still more certain that other unknown enzymes, which have not been identified and studied, must exist, so that the following grouping must be regarded as one which will have to be altered from time to time as additional information is obtained.

CLASSIFICATION OF ENZYMES

	Substrates	End Products
A. Hydrolyzing enzymes		
I. Esterases		
1. Lipases		
a. Glyceridases	Glycerides	Glycerol + fatty acids
b. Others (uncertain)	Fatty acid esters of alcohols other than glycerol	Alcohol + fatty acids
2. Chlorophyllase	Chlorophyll *a*	Chlorophyllide *a* + phytol
3. Pectase	Pectin	Pectic acid + MeOH
4. Cholesterase	Cholesterol esters	Cholesterol + R COOH
5. Tannase	Tannin	Glucose + gallic acid
6. Phosphatases		
a. Lecithinase	Lecithin	Choline + H_3PO_4 + fat
b. Hexosediphosphatase	Hexosediphosphoric acid	Hexose + H_3PO_4
c. Phosphatese (synthesizing)	Hexose + H_3PO_4	Hexosephosphate
d. Polynucleotidase	Nucleic acid	Mono-nucleotides
e. Phosphonuclease	Nucleic acid	Mono-nucleosides + H_3PO_4
f. Phytase	Phytin	Inositol + H_3PO_4
7. Sulfatase	Phenol sulfates	Phenol + $KHSO_4$
II. Carbohydrases		
1. Fructosidases (sucrase)	(a) Sucrose	Fructose + glucose
	(b) Raffinose	Fructose + melibiose
	(c) Gentianose	Fructose + gentiobiose
	(d) Stachyose	Fructose + mannotrisaccharide
2. α-Glucosidases		
a. Maltase	Maltose	Glucose
b. Trehalase	Trehalose	Glucose
c. Others (uncertain)	α-Glucosides	Glucose + non-sugar

CLASSIFICATION OF ENZYMES—*Continued*

	Substrates	End Products
3. β-Glucosidases		
a. Emulsin (mixture)	All β-Glucosides	Sugar + non-sugar residue
b. Amygdalase	Amygdalin	Glucose + prunasin
c. Prunase	Prunasin	Glucose + *d*-mandelonitrile
d. Cellobiase	Cellobiose	Glucose
e. Gentiobiase	Gentiobiose	Glucose
4. Other glucosidases		
a. Oxynitrilase	Oxynitrile	Aldehyde + HCN
b. Vicianase	Vicianin	Benzaldehyde + HCN + gluco-arabinose
c. Robininase (rhamnodiastase)	Robinine	Camphor + robinose
5. β-Galactosidases		
a. Lactase	Lactose	Galactose + glucose
b. Melibiase	Melibiose	Galactose + glucose
6. Amylase (liquefying)	Insoluble starch	Soluble starch
7. α-Amylase (saccharifying)	Soluble starch	α-Maltose
8. β-Amylase (saccharifying)	Soluble starch	β-Maltose
9. Cellulase	Cellulose	Cellobiose
10. Hemicellulases (cytase)	Hemicelluloses	Simple sugars
11. Lichenase	Lichenin	Cellobiose
12. Inulase	Inulin	Fructose
13. Protopectinase	Protopectin	Pectin
14. Pectinase	Pectic acid	Galactose + galacturonic acid
III. Enzymes hydrolyzing nitrogen compounds		
1. Proteases		
a. Rennin	Casein	Paracasein
b. Pepsin	Native proteins	Proteoses and peptones
c. Trypsin	Native proteins	Polypeptides and amino acids
d. Erepsin	Polypeptides	Amino acids
e. Papain	Native proteins	Polypeptides and dipeptides
f. Bromelin	Native proteins	Polypeptides and dipeptides
2. Desamidases		
a. Urease	Urea	Carbon dioxide + ammonia
b. Asparaginase	Asparagine	Aspartic acid + ammonia
c. Arginase	Arginine	Urea + ornithine
d. Histozyme	Hippuric acid	Benzoic acid + glycocoll

CLASSIFICATION OF ENZYMES—*Continued*

	Substrates	End Products
e. Guanosin desamidase	Guanosine	Guanine + pentose
f. Adenosin desamidase	Adenosine	Adenine + pentose
g. Xanthosinase	Xanthosine	Xanthine + pentose
h. Inosinase	Inosine	Hypoxanthine + pentose
i. Guanase	Guanine	Xanthine + ammonia
j. Adenase	Adenine	Hypoxanthine + ammonia
B. Desmolyzing enzymes		
I. Zymase	Hexose	Ethanol + carbon dioxide
II. Glycolase	Hexose	Lactic acid
III. Decarboxylase	$R \cdot CO \cdot COOH$	$R \cdot CHO + CO_2$
IV. Enzymes involved in oxidation-reduction		
1. Alcoholoxidase	Ethanol	Acetaldehyde
2. Purinoxidases	(a) Hypoxanthine	Xanthine
	(b) Xanthine	Uric acid
	(c) Uric acid	Allantoin + carbon dioxide
3. Oxidoreductase	$R \cdot CHO$	$R \cdot CH_2OH + R \cdot COOH$
4. Glyoxalase	Methylglyoxal	Lactic acid
5. Reductases	(a) Methylene blue	Leuco base
	(b) Nitrates	Nitrites
6. Phenolases	Phenols	Quinones
a. Laccase	Lac	Lacquer
b. Tyrosinase	Tyrosine	Black pigments
c. Dopaoxidase	Dopa (dihydroxyphenylalanine)	Black pigments
7. Luciferase	Luciferin	Oxyluciferin and light produced by bioluminescent organisms
V. Catalase	H_2O_2	$H_2O + O_2$

THE CHEMICAL NATURE OF ENZYMES.—No one has ever obtained a product which has been generally accepted by workers in the field of enzyme chemistry as a pure enzyme. Until the last few years the belief has been rather general that enzymes were protein in nature. Thus, Sherman[13] states rather definitely that both pancreatic and malt

[13] Sherman, H. C., The Chemical Nature of Two Typical Enzymes.—Pancreatic and Malt Amylases, *Proc. Nat. Acad. Sci.*, 9 : 81–86 (1923).

amylases are of a protein nature. However, the more recent extensive investigations of Willstätter and his coworkers have thrown doubt on this belief. Willstätter's viewpoint has likewise been accepted by Oppenheimer[14] who makes the statement, (p. 15) " The purest enzyme preparations which we know are those of Willstätter. These belong to none of the usual groups, proteins, carbohydrates, etc. What they are, we know not. Our knowledge shrinks to the generalization that they are demonstrable only by their activity."

Both Willstätter and Oppenheimer take the viewpoint that enzymes are amphoteric electrolytes which exist in a particular colloidal state. Willstätter[15] discusses this question at considerable length, the conclusion being that enzymes contain a special reactive group which either combines with, or possesses some particular affinity for, definite groupings in the substrate, thus accounting for the specificity of enzyme behavior. This special reactive group is attached to a colloidal carrier, and enzyme action is determined in part by the affinity of the active group for the substrate and in part by the colloidality of the entire aggregate. He notes that when the colloidal properties of the aggregate are destroyed, then the activity of the enzyme likewise disappears. Willstätter's view of a specific reactive group combined with colloidal properties appears to be as far as we can generalize with our present knowledge, inasmuch as it affords a synthesis for two opposing schools of thought. The one school is typified by the writings of Fodor[16] where the hypothesis has been put forth that enzymes are nothing more nor less than commonly known substances, proteins, carbohydrates, etc., in a peculiar colloidal state, the activity being due to the energies characteristic of colloid systems. The other, purely chemical school of thought has postulated that enzymes are specific and definite chemical compounds analogous to the hormones, and has endeavored to isolate pure chemical compounds which would be the pure enzymes.

The difficulty in determining the nature of enzymes lies in the fact that they exist in the colloidal state and are accompanied in their extracts by many other colloidal and non-enzymatic substances. A very great concentration of enzymatic activity can often be brought about by differentially adsorbing the enzymes upon precipitates. Willstätter has made extensive use of this adsorption technic with subsequent elutria-

[14] Oppenheimer, C., Die Fermente und ihre Wirkungen, Vol. I, Fifth Edition, Georg Thieme, Leipzig (1925).

[15] Willstätter, R., Graser, J., and Kuhn, R., Zur Kenntnis des Invertins, *Z. physiol. Chem.*, 123 : 1–78 (1922). Note particularly pages 45–60.

[16] Fodor, A., Das Fermentproblem, Theodor Steinkopff, Dresden and Leipzig, 1922.

tion, the adsorption generally occurring at one hydrogen ion concentration, and the release from the adsorbing surface at an altered hydrogen ion concentration. In this way enzymatic preparations which are exceedingly active have been prepared. Unfortunately, this method, while successful in effecting marked concentration, eventually fails, apparently because certain of the colloidal impurities which are present " protect " the enzyme against inactivation. A definite point in purification is therefore reached, past which further attempts at purification result in a loss of activity. Commercial preparations of enzymes are notoriously impure. Thus, Marston[17] was able to concentrate 70 per cent of the activity of " trypsin puriss. sicc. Grübler " into 2 per cent of the preparation by a single precipitation of the enzyme with the dyestuff, safranine.

Sumner[18-20] claims to have isolated the enzyme, urease, in the pure form. The preparation was isolated from jack bean meal. He identifies it as a globulin, crystallizing in colorless octahedra. At least a part of his argument that this crystalline globulin is the pure enzyme is based upon his observation that a given weight of the crystals has the same activity following a recrystallization. There is no doubt but that Sumner has isolated an enzyme preparation of very high activity. The urease preparation which he studied had an activity of 129,000 units, *i.e.*, 1 gram will produce 129,000 milligrams of ammonia nitrogen from a urea phosphate solution in five minutes at 20° C., or it will decompose its own weight of urea in less than 1.4 seconds. Sumner notes that prior to his isolation of this crystalline globulin, the most active preparation of urease was equivalent to only 30,000 units of ammonia nitrogen per gram of protein which was present. Sumner's preparation is destroyedby heat and very rapidly destroyed by exposure to pure water. It is protected by a number of colloid materials and appears to be completely protected for several hours by gum arabic. No one as yet has reported a confirmation of Sumner's crystalline urease. Even in the event that confirmation were reported, the possibility still arises that the enzyme, urease, is present as an adsorbed impurity upon a crystallizable but inert globulin surface. This apparently is the viewpoint which has been taken by Willstätter.

[17] Marston, H. R., The Azine and Azonium Compounds of the Proteolytic Enzymes, I., *Biochem. J.*, 17 : 851–859 (1923).

[18] Sumner, J. B., The Isolation and Crystallization of the Enzyme Urease, Preliminary Paper, *J. Biol. Chem.*, 69 : 435–441 (1926).

[19] Sumner, J. B., Note. The Recrystallization of Urease, *J. Biol. Chem.*, 70 : 97–98 (1926).

[20] Sumner, J. B., and Hand, D. B., Crystalline Urease. II., *J. Biol. Chem.*, 76: 149–162 (1928).

Inasmuch as enzymes are known only by their activity, we may well conclude this section with a free translation[21] from Oppenheimer. "All that we know of the nature of enzymes, and even our very meager first insight into a future structural chemistry and its apparent dependency upon colloid chemical behavior can only be measured by their activity, and the classification of enzymes is likewise based upon this same criterion. In enzyme classification there is no hint of the chemical structure of the enzymes or of the interrelation of specific chemical groupings and the reactions which occur. One must again reiterate that from the purely chemical viewpoint, there is no possibility of arriving at a definition of enzymes, for the definition deals only with activity, the activity of a catalyst produced by living organisms."

THE KINETICS OF ENZYME ACTION.—The law of mass action involving the transformation of one compound into another states that the rate of reaction must be proportional to the concentration of the reacting molecules. Thus, if a molecule of maltose is undergoing the process of hydrolysis, giving rise to two molecules of glucose, the rate of hydrolysis must be proportional to the concentration of the maltose which is present and to the amount of glucose which has already been formed. Inasmuch as such reactions ordinarily take place in the presence of a great excess of water, the change in the concentration of the water is usually ignored, and while one molecule of water is taken up for each molecule of sugar which is hydrolyzed, the small amount of water which is used does not appreciably alter the water concentration. The transformation of a molecule of maltose by hydrolysis into two molecules of glucose is what is known as a monomolecular reaction. Such a reaction may be expressed by the equation

$$v = \frac{dx}{dt} = K(a-x) \qquad (127)$$

where v = the rate of the reaction at time, (t).
x = the amount of substance which has already been transformed in time, (t).

[21] "Alles, was wir von Fermenten wissen, von den ganz spärlichen ersten Hinweisen auf eine zukünftige Strukturchemie (Euler, Willstätter) und ihren offensichtlichen Zusammenhängen mit kolloidchemischen Problemen abgesehen, ist immer gemessen an der Wirksamkeit, und das 'natürliche System' der Fermente, das wir finden werden, ist wiederum auf solchen Kriterien basiert.

"Darin fehlt nun jeder Hinweis auf chemische Strukturen und ihre Zusammenhänge. Es muss tatsächlich mit voller Offenheit ausgesprochen werden: Rein chemisch fehlt uns jede Möglichkeit, die Fermente irgendwie zu definieren. Denn die Definition trifft nur die Wirkung, eben die eines biologisch erzeugten Katalysators."

$(a—x)$ = the concentration of the material which is being transformed, (a) being the initial concentration of the substance.

K = a constant.

This equation states that *the rate of reaction is proportional to the concentration at any specific time.* It has been abundantly demonstrated that an equation of this type fits the usual hydrolytic reactions, such as are involved in the catalytic inversion of cane sugar in the presence of hydrogen ions, the catalytic inversion of maltose, lactose, etc. By integrating equation (127) we obtain the equation,

$$K = \frac{1}{t} \log_e \frac{a}{(a - x)} \tag{128}$$

which can again be expressed as,

$$K = \frac{1}{(t_2 - t_1)} \log_e \frac{C_1}{C_2} \tag{129}$$

where C_1 and C_2 = the concentrations of the substrate at time (t_1) and (t_2) respectively, the time being taken from the beginning of the reaction.

It will be noted that in the last two equations we are dealing with a logarithmic curve where one set of coordinates is a series of numbers and the other set is the logarithms of numbers.

Equation (129) makes it possible to determine the velocity constant in the original equation (127) from any two determinations of the concentration of the substrate at any two intervals of time.

The validity of these monomolecular reaction equations has been demonstrated for certain enzymatic reactions. The author believes that most of the discrepancies between the experimental and theoretical (calculated) data based upon a monomolecular formula are due not to the actual divergence of the reaction from a monomolecular reaction but rather to uncontrolled, and in many instances uncontrollable changes which occur in the system under investigation. That is to say, if all variables excepting the concentration of the substrate could be held constant over the range t_0 to t_1 to t_2, it is entirely probable that the monomolecular laws would be found to hold rigidly. However, it is impossible to demonstrate that there has been no alteration in the activity of the enzyme preparation during the course of the experiment. If the activity of the enzyme is increased, there will be a deviation from the expected results. If any proportion of the enzyme is inactivated or destroyed, this again will cause a deviation from the theoretical values. In studies where we deal only with the concentration of hydrogen ions, it is possible

to set up a univariant system. No biological system as yet realized is absolutely univariant, and until such systems can be set up and accurately controlled, we must expect at the best to reach only approximations of the theoretical results.

One additional factor comes into play in dealing with enzymatic reactions. We have already noted that in many instances hydrolysis and synthesis may be expressed by a reversible reaction and have already set up the hydrolytic equilibrium,

$$\text{Glucoside} + \text{water} \leftrightharpoons \text{sugar} + \text{non-sugar}.$$

The reaction reading from left to right is a monomolecular reaction. The reaction reading from right to left is a bimolecular reaction. The velocity constant for the synthetic reaction will not be identical with that for the hydrolytic reaction. The synthetic reaction may be expressed as

$$\frac{dx}{dt} = K_2 x^2 \tag{130}$$

where x = the concentration of either the uncombined sugar or the uncombined non-sugar radical at time (t).

Inasmuch as these were originally present in equivalent quantities and they combine in equal proportions, the concentration of each can be designated by the same symbol. Accordingly if any tendency toward a synthetic reaction occurs when enzyme phenomena are under investigation, it would be necessary to provide for such a possibility by including in the equation both the hydrolytic and the synthetic velocity constants. Such a possibility has been discussed by Moore,[22] who suggests the equation,

$$\frac{dx}{dt} = K_1(a - x) - K_2 x^2 \tag{131}$$

where K_1 = the velocity constant of the hydrolytic process, and
K_2 = the velocity constant of the synthetic process.

Moore goes further than this and adds to the equation an expression for any change which may have taken place in the activity of the enzyme preparation during the time of the experiment. However, inasmuch as it is extremely difficult to demonstrate the rate of alteration of enzymatic

[22] Moore, B., Velocity of Reaction, and the Comparative Action of Enzymes and Cells—Experimental Observations on Velocity of Reaction, and Their Discussion—Alteration of Concentration of Enzyme, Chapter III of Recent Advances in Physiology and Bio-Chemistry, edited by Leonard Hill, Edward Arnold, London (1908).

activity, we will not include his expressions at this point, the reader being referred to his papers for that discussion.

The above discussion has been based upon the assumption that enzymatic reactions obey the physico-chemical laws of true solutions. As we have already noted in the discussion of the colloid state, our knowledge of the energies of colloid systems is in most instances too inadequate to draw final conclusions and to fit colloidal behavior completely into the scheme of the classical physico-chemical systems. Accordingly it is not surprising that most workers have found enzymatic reactions to deviate somewhat from the theoretical as calculated by the above equations.

Rate of diffusion undoubtedly plays a very important rôle in any system of chemical kinetics. When we are dealing with colloidal systems or when colloidal phases are present, the rate of diffusion of the colloid may be infinitely low, so that only one of the components of the reaction diffuses at an appreciable rate. Thus, if Willstätter's views are correct, *i.e.*, that enzymes possess a chemically reactive group, we must expect the reactions taking place in such a system to be somewhat slowed down, inasmuch as the molecule which is being hydrolyzed must diffuse to the reactive group, and after hydrolysis has taken place, the hydrolytic products must diffuse out from the reactive group in order to allow additional substrate to come in contact at the interface. This factor alone may be sufficient to account for some of the divergencies which have been observed in enzyme studies.

Adding to this the possibility that the hydrolytic products, or the substrate itself, or even the water which makes up the dispersions medium may alter continuously or discontinuously the colloidality of the enzyme itself, only illustrates the complexity of the problem of enzyme kinetics. We have emphasized again and again the ease with which colloidal systems may undergo profound alteration. It is not surprising, therefore, that the environment of the enzyme profoundly affects not only the rate of reaction but the maintenance or the rapid destruction of enzyme activity. Accordingly the presence or absence of traces of various substances may profoundly change the course of an enzyme reaction. A few instances of this sort will be discussed in the succeeding sections. Presumably it is the maintenance or the destruction of the colloidality of the carrier of the reactive group which is influenced.

In the study of rates of reactions in homogeneous systems, great emphasis has been laid upon the fact that reliable results cannot be obtained except when all factors are held constant excepting the one variable which is being studied. Unfortunately we have not yet reached the

point in enzyme chemistry where we can realize this ideal. However, it should be the goal toward which we should strive, and insofar as possible enzyme studies should be conducted with the viewpoint of studying systems possessing only a single variable. The importance of certain of the more significant variables is noted in the succeeding sections.

Hydrogen Ion Concentration.—Inasmuch as enzymes appear to have the properties of lyophilic colloids, it might be expected that their activity would be profoundly influenced by hydrogen ion concentration. This expectation agrees with experimental findings. Enzyme preparations, in general, show a definite optimum hydrogen ion concentration at which the rate of reaction may be many times greater than at other

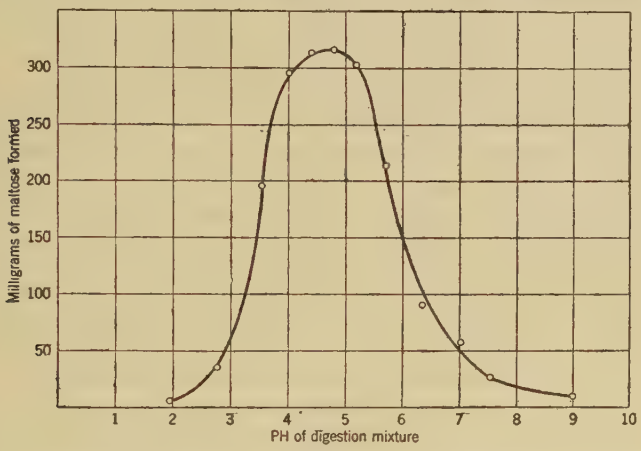


Fig. 129.—Showing the influence of hydrogen ion concentration on the activity of wheat flour amylase. 10 grams of wheat flour autodigested at 27° for one hour. (Data of Rumsey.)

different hydrogen ion concentrations. Thus, the proteolytic enzyme, pepsin, shows its optimum activity in a highly acid solution approximating a *p*H of 2.0. Trypsin, on the other hand, has its greatest activity in a nearly neutral or faintly alkaline solution, approximately *p*H 8.0. Figure 129, taken from the data of Rumsey,[23] shows the relationship between the activity of the amylases of wheat flour and the hydrogen ion concentration of the medium. In these experiments ten-gram samples of wheat flour were added to 100 cc. of distilled water which had already been brought to temperature, and the samples were allowed to digest at 27° C. for one hour. It will be noted that there is an increase of more

[23] Rumsey. L. A., The Diastatic Enzymes of Wheat Flour and Their Relation to Flour Strength, Ph.D. thesis, University of Minnesota, published as Bulletin No. 8, The American Institute of Baking (1922).

than 500 per cent in the rate of reaction at a *p*H of 5.00, as contrasted to the activity at *p*H 7.00. Similar curves have been plotted by various workers for many of the enzymes. In general, the form of the curve is very similar to that shown in Fig. 129, although the point at which the maximum activity is reached may be shifted, depending upon the enzyme which is being studied.

Particular attention should be called to the extremely steep slope of the curve away from the optimum area on both the ascending and descending sides. It is evident that a very slight change in hydrogen ion concentration on this portion of the curve might readily account for very great changes in activity.

Time.—In any biological system, time must be considered as of equal importance to any of the other variables. This fact has been either overlooked or ignored by many workers in the field of enzyme chemistry. Thus, for example, we read that some particular enzyme has a given "optimum" hydrogen ion concentration, or that a given enzyme has an "optimum" temperature, or that it has an "optimum" for some other variable. The author wishes definitely to challenge every statement which has been made in regard to any "optimum condition" in which the variable, *time*, is not stated. *There can be no optimum hydrogen ion concentration and no optimum temperature independent of time.* This is evident when we consider that every enzyme reaction taking place is a balanced reaction, the balance being between enzyme action and enzyme destruction. A given amount of enzyme is present at the beginning of the experiment. This amount of enzyme may well have changed during the course of the experiment. The rate of increase of enzyme activity and the rate of increase of enzyme destruction, insofar as we know, bear no constant relationship. Accordingly, a change to one hydrogen ion concentration may greatly speed up enzymatic activity and only slightly alter the rate of enzyme destruction. Changing to another hydrogen ion concentration may maintain the same enzymatic activity or may cause a slight increase in activity at the same time greatly increasing the rate of enzyme destruction, so that the experimental results would indicate a decrease of enzyme action not due to a decreased enzyme activity but rather to an increased enzyme destruction. Inasmuch as we are always dealing with these two opposing forces, any optimum must be defined in terms of time.

Many of our textbooks are full of statements that the optimum temperature for enzymatic activity is approximately 37° C. This is probably true for pepsin or trypsin, and for most of the enzymes obtained from warm-blooded animal sources, providing that time is measured in hours. On the other hand, if time is measured in days, it may well be

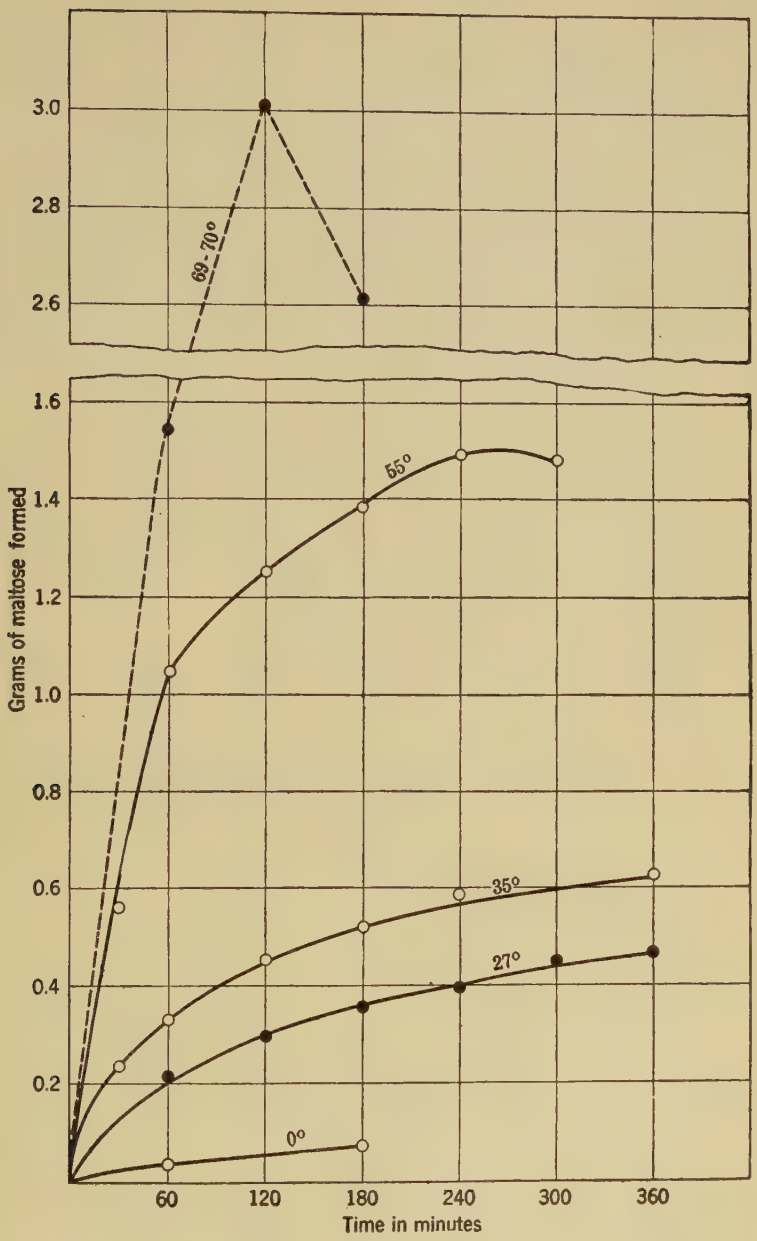


FIG. 130.—Showing the maltose formed by wheat flour amylase acting upon raw wheat starch, with time and temperature as variables. (Data of Rumsey.)

that the optimum temperature will approximate room temperature; or if time is measured in weeks, it may well be that the optimum temperature may approximate the ice-box temperature; or if it is measured in minutes, it is not improbable that the optimum temperature may lie at 50°, 60°, or even 70° C.

Most enzymatic studies dealing with the hydrolysis of starch by amylases have been conducted at incubator temperature somewhere in the neighborhood of 37° C. In many instances, the time has been measured in hours. Figure 130, again taken from the data of Rumsey, shows the combined effects of temperature and time upon the autodigestion of ten grams of wheat flour suspended in 100 cc. of distilled water. This

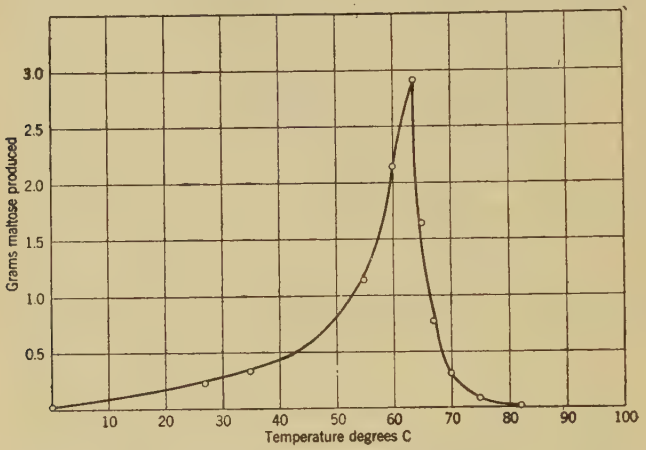


FIG. 131.—Showing the effect of temperature at constant time (1 hour) on maltose production from the autodigestion of 10 grams of wheat flour. (Data of Rumsey.)

suspension had a *p*H of 5.7. Particular attention is directed to the curve showing the rate of maltose production at 50° C. It will be noted that the initial part of this curve is almost perpendicular, that after about thirty minutes the curve begins to flatten out, and at the end of one hundred and twenty minutes has reached approximately 90 per cent of the height which is reached at the end of three hundred and sixty minutes. In other words, the curve for the saccharogenic activity of the wheat flour amylases is an expression of both temperature and time. The activity of the enzyme is enormously higher at 55°, but apparently enzyme destruction likewise proceeds very rapidly at this temperature, resulting in the inactivation of the enzyme which is present.

This is perhaps more strikingly shown in Fig. 131, where time has been held constant and temperature is the only variable. Time in this fig-

ure is one hour, the other conditions of the experiment being as already noted. Certainly in this experiment the optimum temperature for the activity of the saccharogenic enzymes present in wheat flour approximates 60° C. However, immediately following this optimum temperature, there is a rapid fall in activity, indicating that enzyme inactivation is progressing at an incomparably greater rate of speed than determines the rate of starch hydrolysis, *i.e.*, the enzyme is being inactivated before it can bring about any appreciable hydrolysis of the starch.

Other and similar curves could be given, where hydrogen ion concentration represents the only variable, and here again we would find different optimum hydrogen ion concentrations for different times and for different temperatures. Sufficient illustrations have been given, however, to emphasize the fact that time must be regarded as one of the most important variables in enzyme studies.

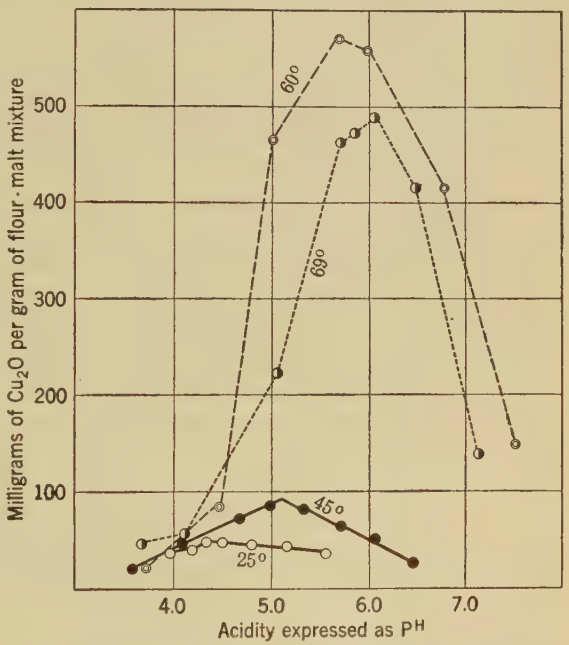


FIG. 132.—Showing the influence of temperature on the optimum hydrogen ion concentration for the saccharogenic activity of malt. Time, 1 hr. (Data of Olsen and Fine.)

TEMPERATURE. — We have noted in the preceding section a number of illustrations which in themselves are sufficient to emphasize the importance of temperature control in enzyme studies. Here again we must recognize that a change in temperature may well cause a shifting of the equilibria in respect to other environmental factors. This is particularly emphasized in the effect of temperature upon optimum hydrogen ion concentration. Olsen and Fine[24] have studied the influence of

[24] Olsen, A. G., and Fine, M. S., Influence of Temperature on Optimum Hydrogen-ion Concentration for the Diastatic Activity of Malt, *Cereal Chem.*, 1 : 215–221 (1924).

temperature upon the optimum hydrogen ion concentration for the activity of the amylase of malt, and Fig. 132 is taken from their data. It will be noted that there are apparently optimum hydrogen ion concentrations of approximately *p*H 6.0 at 70°, approximately *p*H 5.0 at 45°, and approximately *p*H 4.3 at 25°. We have already noted in Fig. 131 the effect of temperature upon the activity of the saccharogenic enzymes present in wheat flour. Collatz[25] found somewhat similar relationships between saccharogenic activity of malt flour and tem-

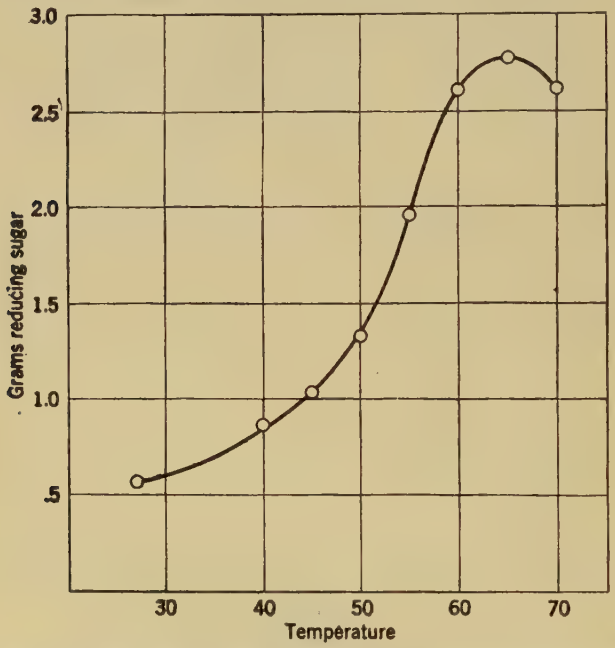


Fig. 133.—Showing the effect of temperature upon the activity of malt amylase when 10-gram portions of malt flour were autodigested for one hour. (Data of Collatz.)

perature. One of his curves is shown in Fig. 133. In this curve, ten grams of malt flour, suspended in 100 cc. of distilled water, were allowed to undergo autodigestion for a period of one hour. The curve shows several distinct portions. An almost linear relationship between temperature and diastatic activity is shown in the area between 27° C. and 45° C.; the rate of activity greatly increases between 45° C. and 50° C.,

[25] Collatz, F. A., Flour Strength as Influenced by the Addition of Diastatic Ferments, Ph.D. thesis, University of Minnesota, published as Bulletin No. 9, The American Institute of Baking (1922).

becomes almost linear between 50° C. and 60° C., still maintaining its increased activity, whereas at approximately 60° C. the activity begins to fall off very rapidly, and at temperatures above 65° C. the rate of enzyme destruction exceeds the rate of starch hydrolysis. Had a different period of time than one hour been chosen, the characteristics of the curve would probably have been altered.

The fact that enzymes may possess extremely great activity at elevated temperatures is not generally emphasized. There are instances, however, where individual usage may be made of this fact. The author calls to mind one instance in the sorghum syrup industry.[26] A certain sorghum syrup factory experienced difficulty in filtration of the defecated juice, due to the presence of traces of starch in the cane which became gelatinized when the juice was heated in the defecators. This gelatinized starch then passed with the juice to the filter presses, causing a clogging of the presses. The clogging of the presses could be prevented only by the use of relatively large quantities of infusorial earth. It was impracticable from the commercial standpoint to cool the juice after defecation to temperatures such as are ordinarily used for the hydrolysis of starch by means of enzymes. Accordingly it was decided to attempt to bring about the hydrolysis of the starch in the defecated juice by the use of amyloclastic enzymes at temperatures exceeding 60° C. Additions of small amounts of such enzyme preparations solved the problem, for although they were rapidly inactivated by the high temperature, the small amount of starch which was present was hydrolyzed, at least to the extent that no further trouble in filtration was experienced. Following the adoption of such a procedure, it was possible to dispense with more than half of the filter presses which had been previously employed and to reduce the amount of infusorial earth which was used, to a fraction of the amount which had previously been required.

The temperature coefficient for enzyme action follows more or less the Van't Hoff rule that the activity is doubled for a 10° rise in temperature. Unfortunately, most of the studies which are available cover only a relatively small temperature range, and in many instances the values compiled from the work of different authors can not be accurately interpreted because of the failure of the authors to record the hydrogen ion concentration or other factors characteristic of the systems which they were studying. Values for Q_{10} as high as 5.30 have been reported for trypsin, and as low as 1.20 for certain of the lipases. These probably represent the extreme range for temperature coefficients, except near the temperature where inactivation is exceedingly rapid.

[26] Willaman, J. J., and Davison, F. R., Starch in Sorghum Sirup, *Ind. Eng. Chem.*, 16 : 609–610 (1924).

Activation and Inactivation of Enzymes.—The literature of enzyme chemistry is replete with papers dealing with the influence of traces of added materials upon enzymatic activity. Perhaps the most consistent series of experiments are those which have been carried out by Sherman[27-35] and his coworkers. Sherman finds, in general, that amino acids protect malt amylase and pancreatic amylase from inactivation, and interprets his results from the viewpoint that these enzymes are protein compounds and accordingly the presence of amino acids in the digest should inhibit the destruction of the enzyme, if the destruction obeys the mass action equilibrium,

$$\text{Protein} + \text{water} \leftrightharpoons \text{amino acids}.$$

Sherman accordingly believes that the protective action of amino acids, in the instances which he has studied, can be interpreted as the retardation of enzyme hydrolysis.

The evidence which Sherman has presented in his papers appears to substantiate his argument. On the other hand, it is difficult to believe that the inhibition of hydrolysis is the sole factor which is involved. Perhaps the most striking of the recent work are the observations by Nord and Franke[36-37] that minute traces of ethylene gas dissolved in

[27] Sherman, H. C., and Walker, F., Influence of Aspartic Acid and Asparagin upon the Enzymic Hydrolysis of Starch, *J. Am. Chem. Soc.*, 41 : 1866–1873 (1919).

[28] Sherman, H. C., and Wayman, M., Effect of Certain Antiseptics upon the Activity of Amylases, *J. Am. Chem. Soc.*, 43 : 2454–2461 (1921)

[29] Sherman, H. C., and Walker, F., The Influence of Certain Amino Acids upon the Enzymic Hydrolysis of Starch, *J. Am. Chem. Soc.*, 43 : 2461–2469 (1921).

[30] Sherman, H. C., and Caldwell, M. L., A Study of the Influence of Arginine, Histidine, Tryptophane and Cystine upon the Hydrolysis of Starch by Purified Pancreatic Amylase, *J. Am. Chem. Soc.*, 43 : 2469–2476 (1921).

[31] Sherman, H. C., and Caldwell, M. L., Influence of Amino Acid in Protecting Amylase from Inactivation by Mercury, *J. Am. Chem. Soc.*, 44 : 2923–2926 (1922).

[32] Sherman, H. C., and Caldwell, M. L., Influence of Lysine upon the Hydrolysis of Starch by Purified Pancreatic Amylase, *J. Am. Chem. Soc.*, 44 : 2926–2930 (1922).

[33] Sherman, H. C., and Naylor, N. M., Influence of Some Organic Compounds upon the Hydrolysis of Starch by Salivary and Pancreatic Amylases, *J. Am. Chem. Soc.*, 44 : 2957–2966 (1922).

[34] Sherman, H. C., and Walker, F., Effect of Amino Acids in Retarding the Hydrolytic Decomposition of an Enzyme (Pancreatic Amylase), *J. Am. Chem. Soc.*, 45 : 1960–1964 (1923).

[35] Sherman, H. C., Caldwell, M. L., and Naylor, N. M., Influence of Tryptophane and Other Amino Acids upon the Stability and Enzymic Activity of Pancreatic Amylase, *J. Am. Chem. Soc.*, 47 : 1702–1709 (1925).

[36] Nord, F. F., and Franke, K. W., On the Mechanism of Enzyme Action. I. A Study of Zymase Fermentation with Contributions to a Theory of Enzyme "Activation," *Protoplasma*, 4 : 547–595 (1928).

water protect the fermentation enzymes of yeast against inactivation. They were able to markedly increase the activity of zymase preparations by bubbling ethylene through the solution and were able to demonstrate that solutions treated with ethylene maintained for a long period of time their ability to ferment glucose. The active fermenting system of zymase and sugar did not bring about the rapid inactivation of the enzyme which was present. Accordingly, Nord and Franke suggest that ethylene acts in this instance not as an activator but rather as a protector, and that in all probability the various experiments in the literature which have been reported as dealing with enzyme activation, should be interpreted as experiments dealing with enzyme protection, the apparent increased activity being due to the inhibition of enzyme destruction. This view appears to the author to be a logical one, assuming, of course, that the other physical factors in the environment have been kept constant. Undoubtedly, at least a part of the effect of hydrogen ion concentration is due to an increase or decrease of the effective surface of the colloidal enzyme, but at a fixed hydrogen ion concentration and a fixed salt concentration it would be logical to interpret the effect of substances which activate enzymes in the same way that the action of protective colloids is interpreted.

This viewpoint appears to agree with that of Velluz[38] who studied in particular the probable mechanism whereby small amounts of fatty acids are able to inactivate pepsin acting upon coagulated egg albumin. Table LXI shows certain of the results which Velluz obtained. In the third column of the table are listed Langmuir's calculations of the surface areas covered by one molecule of the fatty acid. Velluz notes that, in general, there is a relationship between the effect of the fatty acid in inhibiting proteolytic activity and the area which is covered by a molecule of the fatty acid, and he notes that pepsin is apparently unable to attack the colloidal micelles of egg albumin when the micelles are partially or entirely covered by an oriented layer of fatty acid molecules. Velluz goes further and tests this hypothesis by adding gum arabic and the fatty acid simultaneously to the pepsin-albumin system and finds that under these conditions there is little or no interference with proteolytic activity. He explains this by assuming that the fatty acid which is added becomes oriented upon the surface of the gum arabic, thus leaving the surface of the protein free to be attacked by the pepsin.

[37] Nord, F. F., and Franke, K. W., On the Mechanism of Enzyme Action. II. Further Evidence Confirming the Observations that Ethylene Increases the Permeability of Cells and Acts as a Protector, *J. Biol. Chem.*, 79 : 27–51 (1928).

[38] Velluz, M. L., Recherches sur l'action inhibitrice des acides gras et, en particulier des acides non saturés, sur les phénomènes diastasiques, *Bull. soc. chim. biol.*, 9 : 483–500 (1927).

TABLE LXI

Showing the Apparent Relationship between the Retardation of Peptic Activity Due to the Addition of Small Amounts of Fatty Acids and the Surface Area Covered by a Molecule of the Fatty Acid (Pepsin 0.25%, HCl 0.25%, Coagulated Egg White 0.5%, Fatty Acid 0.04%, Time 16 Hours). (From Data of Velluz.)

Fatty Acid Added	Soluble Amino Nitrogen Mgs. per 100 cc.	Area Covered by a Molecule of Fatty Acid (after Langmuir), $\times 10^{-16}$ sq. cm.
None	7.2	
Caproic	6.6	
Caprylic	6.3	
Capric	6.1	
Palmitic	5.8	21
Stearic	5.8	22
Oleic	2.8	46
Ricinolic	1.2	110
Linolenic	1.3	65

One other factor should be mentioned in the discussion of activation and inactivation, *i.e.*, the effect of the accumulated end products upon the course of the reaction. This has already been noted in the discussion of the kinetics of enzyme action. It seems logical to assume, at least until demonstrated to the contrary, that all enzyme reactions proceed to an equilibrium. Accordingly as a reaction progresses and the concentration of the end products increases, one should expect according to the law of mass action that the reaction would be slowed up. We have already noted that we know far too little in regard to the nature of enzymes and of the variables which may be present in a given system to introduce necessary corrections to account for this retardation. The problem is complicated enough when we can demonstrate that only a single enzyme is involved. It may well be that our supposedly single enzyme preparation contains in reality a mixture of enzymes. If these enzymes induce different reactions, it may be possible to demonstrate that our enzyme preparation is a mixture. However, it is not inconceivable that different enzymes may induce identical reactions or at least reactions which are essentially indistinguishable from each other by our present methods. That this is a probability is evidenced by the paper of Kuhn and Münch.[39] These authors studied twelve sucrase

[39] Kuhn, R., and Münch, H., Über Gluco-und Fructosaccharase, *Z. physiol. Chem.* **150** : 220–242 (1925).

preparations derived from twelve different strains of yeast, and were able to divide these twelve preparations into four groups. The method employed was to study the influence of added glucose or fructose (the products of hydrolysis of sucrose) upon the rate of sucrose hydrolysis. The four sucrases which they identified were: (1) those sucrases whose rate of reaction was slowed up by the addition of β-glucose much more than by the addition of α-glucose; the addition of fructose likewise slowed up the rate of reaction of this group; (2) those sucrases whose rate of reaction was slowed up by the addition of fructose but whose rate of reaction was not altered by the addition of either α- or β-glucose; (3) those sucrases whose rate of reaction was retarded by the addition of either α- or β-glucose but was not altered by the addition of fructose; and (4) those sucrases whose rate of reaction was altered equally by the addition of either α-glucose, β-glucose, or fructose. Table LXII shows examples of the data of Kuhn and Münch.

TABLE LXII

Showing the Retardation of the Action of Various Saccharase Preparations by the Addition of α-Glucose, β-Glucose, or Fructose (0.2 N Sucrose, 0.2 N Hexose, 25° C., *p*H 5.0). (Data of Kuhn and Münch.)

Saccharase No.	Type	Sugar Added—Retardation		
		α-Glucose, Per Cent	β-Glucose, Per Cent	Fructose, Per Cent
A	I	0.0	23.0	15.5
K	I	0.0	9.2	25.5
B	II	0.0	0.0	10.0
M	II	0.5	0.5	30.0
P	III	9.0	11.5	0.0
F	III	5.9	8.8	0.0
E	IV	26.0	28.0	25.0
D	IV	18.0	18.5	16.5

If the observations of Kuhn and Münch are substantiated by other investigators, it is obvious that a new field in enzyme chemistry has been opened. Possibly such a substantiation may explain the divergences in the viewpoints of various investigators, for obviously if the interpretations of Kuhn and Münch are correct, the literature on "invertase" is filled with papers where one investigator has been discussing the behavior of one enzyme and another investigator the behavior of a different enzyme. The author knows of no comparable data in regard to the intra-classification of the other types of enzymes.

Possibly a study of specific compounds which bring about enzyme inactivation may afford a clue as to the chemical nature of the enzymes themselves. At least this is the viewpoint of Palmer[40] who notes that formaldehyde readily unites with proteins and accordingly might be assumed to have a very pronounced effect upon enzyme action, providing that enzymes possess a protein nature. Palmer found formaldehyde to slightly stimulate lipolysis, and it was not until the concentration of formaldehyde reached approximately 1 per cent that any retarding effect was observed. On the other hand, traces of free iodine almost completely inhibited lipase activity; from which Palmer suggests that lipase probably does not possess a protein nature, but may eventually be found to be more or less analogous to an unsaturated fat or a fatty acid. Free bromine was somewhat less effective than free iodine, although even small quantities of bromine produced marked inactivation. Palmer, by the use of this differential inactivation, was enabled to completely inactivate proteolytic enzymes and maintain without impairment the activity of the lipolytic enzyme, or conversely he was able to completely inactivate the lipolytic enzymes without appreciably altering the activity of the proteases which were present.

Enzyme preparations are readily inactivated by traces of the heavy metals. This is notably true of such metals as copper, silver, mercury, and lead. We have noted that these same metals " poison " the hydrogen electrode, and we have similarly noted that at least three of these metals are the " cumulative poisons " insofar as living organisms are concerned, and further that they have a profound influence upon both lyophilic and lyophobic colloid systems. It seems logical, therefore, to interpret the effect of these metals on the inactivation of enzyme systems in terms of colloid chemistry and to postulate that a pronounced reduction of active surface is brought about by the presence of these metal ions.

Specific Enzyme Reactions.—We have already noted in this chapter that the literature dealing with specific enzymatic reactions is exceedingly voluminous, and that from time to time in the preceding chapters we have discussed certain interrelationships between enzymes and substrates. In view of these facts, it seems wise to close the discussion of enzymes at this point, having emphasized only some of the broader principles which are involved. To extend the discussion so as to make this text adequate in the field of enzymes would be to extend it beyond the purposes for which the volume is intended.

[40] Palmer, L. S., The Influence of Various Antiseptics on the Activity of Lipase, *J. Am. Chem. Soc.*, 44 : 1527–1538 (1922).

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